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(54) Title: NOVEL MEMBERS OF THE LYSYL OXIDASES FAMILY OF AMINE OXIDASES RELATED APPLICATIONS

(57) Abstract: In accordance with the present invention, there are provided additional LOX-like proteins characterized by their structural homology to LOX, LOXL1 and LOXL2, and containing a copper binding domain and a catalytic domain. Nucleic acid sequences encoding LOXL3 and LOXL4 proteins, antibodies derived threfrom, as well as assays employing these proteins for determining tissue distribution and catalytic activites, are also disclosed. Invention proteins can be employed in a variety of ways, such as, for example, in bioassays, for production of antibodies thereto, in therapeutic compositions containing such proteins and/or antibodies. The present invention also discloses methods and diagnostic systems for determining the levels or activities of proteins (or functional fragments thereof) related to the LOX gene family, methods for producing a transgenic animal model comprising disrupted genes encoding proteins (or fragments thereof) related to LOX gene family, and methods for identifying compound(s) which modulate the activity of proteins (or functional fragments thereof) related to the LOX gene family. The present invention therefore, discloses the gene structure, chromosomal localization, protein domain structure, evolutionary relationship, tissue specific expression and distribution of lysyl oxidase (LOX) and LOX-like proteins including but not limited to LOXL, LOXL2, LOXL3 and LOXL4, that defines a family of proteins present in distinct cellular and tissue locations, each with a related but different function.

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NOVEL MEMBERS OF THE LYSYL OXIDASES FAMILY OF AMINE OXIDASES RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/201,587, filed May 3, 2000, entitled LYSYL OXIDASES: A NOVEL FAMILY OF AMINE OXIDASES, which is hereby incorporated by reference in its entirety, including drawings.

FIELD OF THE INVENTION

The present invention relates generally to lysyl oxidases, members of the copper-dependent amine oxidase protein family, DNA sequences encoding same, and various uses therefor.

BACKGROUND OF THE INVENTION

Lysyl oxidase (LOX) belongs to a heterogeneous family of copper-dependent amine oxidases that oxidize primary amine substrates to reactive aldehydes (Janes *et al.*, Biochemistry 31: 12147, 1992; Lyles, Int. J. Biochem. Cell Biol. 28: 259-274, 1996; Dove *et al.*, FEBS Lett. 398: 231-234, 1996). From the time of the discovery of LOX (Pinell and Martine, Proc. Acad. Sci. USA 61: 708-714, 1968) most studies have focused on the specific cross-linking activity and catalytic mechanism of action of this enzyme on collagen and elastin substrates, essential to the biogenesis of connective tissue.

Recently, multiple novel biological functions have been attributed to LOX. Evidence from several laboratories suggests that LOX may have other intracellular or intranuclear substrates involved in these functions. The range of activities attributed to LOX cover a spectrum of biological functions including developmental regulation, tumor suppression, senescence, cell growth control and chemotaxis (Contente *et al.* Science 249: 796-798, 1990; Lazarus *et al.* Matrix Biol. 14: 727-731, 1994; Mello *et al.* Exp. Cell

Res. 220: 374-382, 1995; Csiszar *et al.* Mol. Biol. Reports 23: 97-108, 1996, Saito *et al.* J. Biol. Chem. 272: 8157-8160, 1997; DiDonato *et al.* FEBS Lett. 419: 63-68, 1997).

Lysyl oxidase participates in the critical post-translational modification, essential to the biogenesis of connective tissue, by oxidizing peptidyl lysine in these proteins to α-aminoadipic-δ-semialdehyde or allysine. This peptidyl aldehyde can then spontaneously condense with neighboring amino groups or other peptidyl aldehydes to form covalent cross-links in several fibrillar collagen types and desmosines and isodesmosines in elastin. These different cross-links are essential for the development of collagen fibrils and insoluble elastin, the major protein component of elastic fibers (Eyre *et al.* Ann. Rev. Biochem. 53: 717-748, 1984; Kagan *et al.* In: Molecular Biology and Pathology of Elastic Tissue, (Editors: Mecham and Roberts), Ciba Foundation Symposium Series, 1994; Smith-Mungo and Kagan, Matrix Biol. 16: 387-398, 1998).

A significant role for LOX in cellular transformation and reversion was reported a few years ago by several laboratories. Friedman and co-workers (Science 253: 802, 1991) first described LOX as a '*ras* recision' protein, encoded by an mRNA dramatically reduced in levels upon transformation of 3T3 cells and re-expressed in high abundance following interferon-mediated reversion of these transformed cells (Contente *et al.* Science 249: 796-798, 1990, Kenyon *et al.* Cell Mol. Biol. 5: 206-210, 1991). The upregulation of LOX synthesis in revertant *ras*-transformed cells has since been independently verified by several other laboratories (Krzyzosiak *et al.* Proc. Natl. Acad. Sci. USA 89: 4879-4883, 1992; Hajnal *et al.* Cancer Res. 53: 4670-4675, 1993; Oberhuber *et al.*, Mol. Carcinog. 12: 198-204, 1995). These initial studies were performed in transformed fibroblasts only and though suggesting anti-oncogenic activity for LOX, neither provided a mechanistic basis, nor proved such a function.

Subsequently, several regulatory mechanisms have been identified that result in modulation of LOX expression and which may contribute to its putative tumor suppressor function. Transcriptional regulation of the LOX gene by oncogenic *ras* was reported by

Csiszar *et al.*, (Mol. Biol. Reports 23: 97-108, 1996). TGF-β1 has also been reported to affect LOX mRNA levels, mainly through a post-transcriptional mechanism that results in strong reduction of cell proliferation rates (Gacheru *et al.* J. Cell. Biochem. 65: 395-407, 1997; Feres-Filho *et al.* J. Biol. Chem 270: 30797-30803; 1995 and 271: 6411-6416, 1996). Loss of TGF-β1 regulation of the LOX gene has also been observed in prostate cancer leading to the progressive decrease of the amount of LOX mRNA in prostate tumors (Ren *et al.* Cancer Res. 58: 1285-1290, 1998). The LOX gene has also been shown to be the target of the anti-oncogenic interferon regulatory factor, IRF-1. The activation of the LOX promoter through the IRF response element results in LOX gene up-regulation and consequent tumor cell reversion (Tan *et al.* Cancer Res. 56: 2417-2421, 1996). While the mechanism by which this amine oxidase may mediate tumor suppression and control cell growth still remains unknown, the observation suggests a possible pathway for the up-regulation of LOX gene expression during interferon-induced reversion.

Altered LOX gene expression has been noted in invasive and metastatic prostate and breast cancer. Significant expression of the LOX gene has been observed in normal prostatic epithelial and stromal cells and a progressive decrease in LOX mRNA levels has been shown to be associated with malignant prostatic epithelial cells and with the development of primary prostate tumors. This reduction in LOX mRNA levels, moreover, has been shown to be part of an aberrant response of these tumorigenic cells to TGF-β1 (Ren *et al.* Cancer Res. 58: 1285-1290, 1998). In breast tumors, abundant LOX is observed in myofibroblasts and myoepithelial cells in benign lesions surrounding *in situ* ductal carcinomas. LOX levels decrease dramatically, however, in invasive ductal breast carcinomas (Peyrol *et al.* Am. J. Pathol. 150: 497-507, 1997). Similarly low levels of LOX mRNA and LOX catalytic activity has been reported in a variety of tumor cell lines including melanoma, fibro-and choriosarcoma, rhabdomyosarcoma and *ras*-transformed osteosarcoma cells as a possible result of both transcriptional down-regulation and decrease in mRNA stability (Kuivaniemi *et al.* FEBS Lett. 195: 261-264, 1986; Contente

et al. J. Biol. Chem 268: 18435-18437, 1993; Science 249: 796-798, 1993; Csiszar *et al.* Genomics 16: 401-406, 1993, and Mol. Biol. Reports 23: 97-108, 1996; Hamalainen *et al.* J. Biol. Chem. 270: 21590-21593, 1995).

The above-noted observations with respect to prostate, breast tumors and several tumor cell lines, and the earlier report describing tumor reversion by interferon, indicate a strong association between reduced levels of LOX and tumorigenesis and support the proposed role of LOX as a tumor suppressor. It has also been demonstrated that somatic LOX gene mutations do occur in colon cancer and may be the primary cause of loss of LOX function in colonic tumors (Csiszar *et al.* 2001, accepted for publication by Eur. J. Cancer).

The LOX gene maps to chromosome 5q23 (Hamalainen *et al.* Genomics 11: 508-516, 1991; Mariani *et al.* Matrix 12: 242-248, 1992), a chromosomal locus in which high frequency interstitial and complete losses are recurring anomalies associated with a number of malignancies including colon, esophageal, lung and gastric carcinomas (Wieland *et al.* Ongogene 12: 97-102, 1996; Tamura *et al.* Cancer Res. 56: 612-615, 1996). A significant loss of heterozygosity of this chromosome 5q23 region (including the LOX gene) has been observed in DNA from colon tumors in over 140 unrelated patients with sporadic colon and esophageal carcinoma (Csiszar *et al.* 2001, *Supra*). Moreover, the surviving LOX gene allele in these tumors also contains inactivating mutations. The loss of function of the LOX gene through these inactivating somatic mutations and the consequent loss of a recessive tumor suppressor phenotype in colon and esophageal tumors is direct evidence suggesting that LOX may indeed serve as a tumor suppressor in a manner similar to other tumor suppressor genes known to be important in the pathogenesis of colon and esophageal cancer such as MCC, APC and p53 (Hoops *et al.* Clinics of North America 11: 609-633, 1997).

LOX may act as a tumor suppressor, either through its catalytic function as a copper-dependent amine oxidase or through a new and completely unknown mechanism.

Mutations of LOX in sea urchin development and in xenopus oocytes have been reported. The amine oxidase activity of LOX was shown to increase several-fold during the development of sea urchin embryos and peaked during gastrulation and larva formation. The treatment of developing embryos with β-amino proprionitrile (βAPN), a specific LOX inhibitor, results in developmental arrest at the mesenchymal blastula stage. This striking arrest of embryonic development suggests a critical role for LOX in mesenchyme migration, gastrulation and morphogenesis during sea urchin development (Butler *et al.* Exp. Cell Res. 173: 174-182, 1987).

The intracellular interaction of LOX and *ras* was tested by co-injecting LOX and oncogenic p21-Ha-*ras* into maturing xenopus oocytes. The intracellular presence of LOX in the oocytes inhibits *ras*-dependent oocyte maturation, while βAPN treatment of these injected oocytes successfully abolishes this inhibition of maturation. LOX dependent inhibition, moreover, appears to be mediated by the *de novo* synthesis of a protein (as yet unknown) that actually acts as an inhibitor to block oocyte maturation in a relatively late stage of the maturation pathway that involves not only *ras* but also Erk2 (DiDonato *et al.* FEBS Lett. 419: 63-68 1997).

The study of LOX provides convincing evidence that LOX does much more in multi-cellular organisms than ensure the mechanical integrity of several extracellular matrix polymers. Experiments involving maturing oocytes in particular, point to a novel intracellular function for LOX.

LOX has been shown to be synthesized as a 48 kd pre-proprotein which appears, following intracellular and extracellular processing, as a 32 kd enzyme in the extracellular matrix (Kagan *et al.* In: Molecular Biology and Pathology of Elastic Tissue, (EditorsL Mecham and Roberts), Ciba Foundation Symposium Series, 1994; Cronshaw *et al.* Biochem. J. 306: 279-284, 1995). This mature form of LOX has been shown to be immunolocalized in connective tissue to the periphery of developing elastic fibers and associated with collagen fibrils (Kagan *et al.* J. Cell Biol. 103: 1121-1128, 1986,

Kobayashi *et al.* Br. J. Dermatol. 131: 325-330, 1994). LOX, however, has been shown to be localized within chondrocytes, cultured epithelial cells (Wakasaki *et al.* Lab. Invest. 63: 377-384, 1990) and within the nuclei of rat vascular smooth muscle cells and 3T3 fibroblasts (Li *et at.* Proc. Natl. Acad. Sci. USA 94: 12817-12822, 1997). LOX-dependent alterations in chromatin structure have also been reported (Mello *et al.* Exp. Cell Res. 220: 374-382, 1995). Moreover, Kagan and co-workers have recently described an intra-nuclear LOX-cross-linking activity. A crosslink was observed in the nucleus, which may result in a LOX-mediated loss of positive charge in a possible nuclear substrate (Li *et al.* Proc. Natl. Acad. Sci. USA 94: 12817-12822, 1997). This process is analogous to the acetylation of lysine residues in histones by histone deacetylase, that is well known to correlate with changes in transcription of nucleosomal DNA (Wolffe *et al.* Science 272: 371-372, 1996).

It has established that extracellular matrix amine oxidases are responsible for the catalysis of lysine-derived crosslinks in a variety of collagen types and elastin. However, it is unclear whether a single enzyme or multiple lysyl oxidases are in fact responsible for the deamination of lysine residues from such diverse substrates (Kagan *et al.* J. Biol. Chem 259: 11203-11207, 1983 and Biochem. Biophs. Res. Comm. 115: 186-192, 1983; Eyre *et al.* Biochem 53: 717-748, 1984, Shah *et al.* J. Biol. Chem. 268: 11573-11579, 1993; Nagan *et al.* J. Biol. Chem. 269: 22366-22371, 1994). While multiple isoforms of LOX have been described in bovine aorta, the biological relevance and origin of these isoforms have not reported (Sullivan *et al.* J. Biol. Chem. 257: 13520-13526, 1982; Kuivaniemi *et al.* J. Biol. Chem. 259: 6996-7002, 1984; Williams *et al.* Anal. Biochem. 149: 430-437, 1985). Several other proteins with either LOX activity or LOX-derived crosslinks have also been described, but the relationship of these proteins or glycoproteins to LOX remains unclear (Eyre *et al.* Ann. Rev. Biochem. 53: 717-748, 1984).

The mechanism(s) of how a single LOX protein can fulfill so many different functions is unknown. Alternatively, several different lysyl oxidases may exist that

individually function to perform these roles, currently attributed to a single enzyme. Over the past few years, two lysyl oxidase-related proteins have been described that fulfill all the requirements of being fully functional, but genetically distinct, LOXs that could serve as a family of proteins present in a variety of cellular and tissue locations, each with a related but different function.

The first of these lysyl oxidase-related proteins is called LOL, or lysyl oxidase-like (Kenyon *et al.*, J. Biol. Chem. 268, 18435-18437, 1993; Kim *et al.*, J. Biol. Chem. 279, 7176-7182, 1995). A cDNA sequence comparison of LOL and LOX confirms a significant homology within the carboxy-terminal portion of these proteins. This homology includes a striking conservation of the copper-binding site, the catalytic and the carbonyl co-factor binding site. This domain conservation is also reflected in conservation of exon size and exon-intron boundaries in five of the seven exons in both the LOX and LOL genes encoding these conserved domains. This gene was subsequently mapped to chromosome 15q23 and renamed LOXL (Szabo *et al.* Hum. Genet. 101: 198-200, 1997; Kim *et al.* Clin. Genet. 51: 131-132, 1997). The localization of the LOXL protein to sites of *de novo* fibrosis in the liver has also been reported and co-regulated expression demonstrated for the LOXL gene with the *colIIIA1* gene and the LOX gene with the gene encoding pro-α1(I) collagen. These results suggest different functions for LOX and LOXL (Kim *et al.*, J. Cell. Biochem. 2, 181-188, 1998).

More recently, a second lysyl oxidase-related protein (LOXL2) has been reported in senescent fibroblasts that contain the same carboxy-terminal sequence conservation noted in LOX and LOXL (Saito *et al.* J. Biol. Chem. 272: 8157-8160, 1997). Characterization of LOXL2 protein has facilitated identification of the corresponding gene and confirmation that LOXL2 is indeed another genetically distinct copper-binding protein closely related to LOX (Jourdan Le-Saux *et al.* J. Biol. Chem. 274(18): 12939-12944, 1999). LOXL2 has been mapped to chromosome 8p21 (Jourdan-Le Saux *et al.* Genomics 2: 305-307, 1998). The unique temporal and spatial tissue-specific expression pattern in reproductive tissues and the possible intracellular localization of LOXL2

indicates a role for LOXL2 that is distinctly different from either LOX or LOXL (Jourdan-Le Saux *et al.* J. Biol. Chem. 274 (18), 12939-12944, 1999).

With the proposed existence of a family of lysyl oxidases, each member with possibly specific substrates, tissue locations, probably different cellular or extracellular sites of action and distinct developmentally regulated patterns of expression, it may now be possible to understand and unravel the details of the relationship between the individual lysyl oxidases and the mechanistic basis for the many functions of these proteins that had previously been attributed to just a single protein, LOX.

Elucidating the biochemical basis for the multifunctional nature of lysyl oxidase(s) is important and relevant to a clearer understanding of the development of normal tissue structure and function. Understanding the biology of this family of proteins is, however, also critical to a better insight into the role of lysyl oxidases in a wide range of acquired and heritable diseases.

The role of lysyl oxidase in human disorders, either as a primary determinant of a disease process or as a secondary consequence of other genetic or environmental factors, has never been clear. By unraveling the complex role of lysyl oxidases in tissue structure and function, new and valuable insight can be obtained into the mechanism(s) by which these amino oxidases influence such a variety of disease processes.

The role of LOX in the pathobiology of colon cancer and the probable role for the LOXL proteins in invasive breast and prostate cancer have been described. It is very likely that the tumor suppressor function of lysyl oxidases will be important to other tumors as well. Confirming the tumor suppressor role and providing a mechanistic basis for this function is particularly significant in the case of the LOXL2, since its gene has been mapped within the chromosome 8p21 region that is minimally deleted in most solid tumors (Wu *et al.* Genes Chromosomes Cancer 20: 347-353, 1997; Haggman *et al.* Urology 50: 643-647, 1997; Wagner *et al.* Am. J. Patbol. 151: 753-759, 1997; Jourdan-Le Saux *et al.* Genomics 2: 305-307, 1998). Moreover, given the important role of LOX-

like proteins in human health, the discovery of additional LOX-like genes would be of great benefit.

SUMMARY OF THE INVENTION

In accordance with the present invention, there are provided additional copper-dependent lysyl oxidase-like proteins, nucleic acids encoding same, methods to produce the same, and uses thereof. Invention proteins are referred to herein as LOXL3 and LOXL4. Full length cDNA sequences encoding invention proteins and the deduced amino acid sequences of LOXL3 and LOXL4 are also provided. DNA molecules encoding LOXL3, LOXL4 and other proteins of the LOX gene family are useful, for example, as probes for detecting the presence of these nucleic acids in biological or pathological samples, for identification of additional proteins of the LOX gene family, as coding sequences which can be used for the recombinant expression of invention proteins (or functional fragments thereof), and the like. Fragments of nucleic acids encoding invention proteins can also be employed as primers for PCR amplification of DNA encoding invention proteins, and the like. Invention proteins can be employed in a variety of ways, such as, for example, in bioassays, for production of antibodies thereto, in therapeutic compositions containing such proteins and/or antibodies, and the like.

In accordance with another aspect of the present invention, anti-LOXL3 and anti-LOXL4 antibodies are also provided. LOXL3 and LOXL4 antibodies, as well as other proteins of the LOX gene family and antibodies thereto, are useful for diagnostic assays to determine expression levels and localization of invention proteins and other proteins of the LOX gene family in various tissue samples from either healthy or infirmed subjects. Anti-LOXL3 and anti-LOXL4 antibodies, and antibodies against other LOX gene family proteins can also be used to purify invention proteins, and the like. Moreover, these antibodies are therapeutically useful to counteract or supplement the biological effect of LOX-like proteins *in vivo*.

In accordance with yet another aspect of the present invention, transgenic animals whose genome has a disruption of one or more genes related to the LOX gene family, *e.g.* LOXL, LOXL2, LOXL3, LOXL4, and the like, are provided. Methods for producing such transgenic animals are also provided. The above-described disruption results in an animal exhibiting a disorder as compared to a wild-type animal, and comprises the insertion or deletion of a transgene including a selectable marker sequence, or a point-mutation of a transgene. Such disruption also results in homozyosity or heterozygosity of a gene related to the LOX gene family.

Methods and diagnostic systems for determining expression levels or activities of invention proteins (or fragments thereof) in various samples or subjects are also provided. These diagnostic methods can be used, for example, to diagnose physiological disorders that result from abnormal levels or activity of invention proteins or other proteins in the LOX gene family.

In accordance with still another aspect of the present invention, methods for determining extracellular and intracellular localization of proteins of the LOX gene family are provided. For example, LOXL2, LOXL3, LOXL4, and the like, are identified as membrane-associated proteins. For example, LOXL2 appears at sites where basement membrane is present. Detection of LOXL2 associated with the plasma membrane of cultured cells, suggests that membrane association may significantly modify the structure and consequently the catalytic activity of LOXL2. Methods for determining the membrane association of LOXL3 and LOXL4 and investigating possible similarities with LOXL2 are also provided.

In accordance with a further aspect of the present invention, methods for determining the activity of LOXL2, LOXL3, LOXL4, and the like, are provided. For example, using synthetic peptide substrates, the amine oxidase activity of immunopurified LOXL2 and membrane associated LOXL2 are measured *in vitro*. The inhibition of the amine oxidase activity of these proteins by βAPN is also tested. The β-

strand structures within the cytokine-binding domain of LOXL2 are different from both LOX and LOXL; these structures, together with the predicted transmembrane localization of LOXL2, significantly modify folding of this protein, further suggesting that LOXL2 and also LOXL3, fulfill other functions such as binding of cytokines or interacting with other proteins and that some of these functions can be associated with the basement membrane.

In accordance with a still further aspect of the present invention, methods for evaluating whether proteins of the LOX gene family are associated with the basement membrane are provided. For example, invention methods are used to determine that cell surface bound LOXL2 is functionally associated with basement membrane collagen type IV, and that LOXL2 is structurally associated with the basement membrane. Methods for the similar measurement and evaluation for LOXL3 and LOXL4 are also provided.

In accordance with another aspect of the present invention, methods for determining the function(s) of members of the LOX family of multifunctional amine oxidases are also provided. Invention methods not only provide information for a better understanding of the role of lysyl oxidase(s) within the extracellular matrix, but also define novel functions thereof in intracellular or membrane locations. These novel functions can be relevant not only to normal tissue function but also to a wide range of diseases in which altered lysyl oxidase activity is observed.

In accordance with still another aspect of the present invention, bioassays for identifying compound(s) that modulate the activity of the LOX-like proteins are provided. Such bioassays can be useful for rapidly screening a large number of compounds to determine which compounds modulate the activity of the LOX-like proteins. Invention bioassays can also be employed to identify new substrates for members of the LOX protein family.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 collectively shows a comparison of conserved domains within lysyl oxidase and lysyl oxidase-related proteins. Fig. 1A illustrates the various domains in lysyl oxidase (LOX) and the extent to which these conserved sequences are present in LOXL, LOXL2, LOXL3 and LOXL4 Certain functional domains are indicated; the lengths of the un-shaded bars represent the relative sizes of the individual proteins. Fig. 1B illustrates the amino acid differences between LOX, LOXL, LOXL2, LOXL3 and LOXL4 in the copper binding and catalytic domains.

Figure 2 shows the structure of the genes encoding LOX, LOXL, LOXL2, LOXL3 and LOXL4. Exons encoding conserved domains include: LOX: exons 2, 3, 4, 5, and 6; LOXL: exons 2, 3, 4, 5 and 6; LOXL2: exons 6, 7, 8, 9 and 10; LOXL3: exons 9, 10 and 11; LOXL4: exons 9 to 12; and exons encoding nonconserved domains include: LOX: exon 1, LOXL: exon 1; LOXL2: exons 1, 2, 3, 4 and 5; LOXL3: exons 1, 2, 3, 4, 5, 6, 7, 8 and 12; LOXL4: exons 1, 2, 3, 4, 5, 6, 7, 8; and the exons containing the 3'UTR include: LOX: exon 7; LOXL: exon 7; LOXL2: exon 11; LOXL3: exon 13; LOXL4: exon 13. No significant conservation of sequence within the 3'UTR is noted among the genes. Chromosomal locations for each gene are on the left of the figure.

Figure 3 shows a tree representation of clustering sequence relationship among five members of the lysyl oxidase family. Available coding sequences for LOX, LOXL, LOXL2, LOXL3 and LOXL4 were used for a progressive, pairwise alignment using the GCG program Pileup. The similarities in aligned sequences are represented in this dendrogram to demonstrate the degree of similarity among all the compared sequences. The biphasic clustering of LOX, LOXL, LOXL2, LOXL3 and LOXL4, provides a closer evolutionary relationship among those lysyl oxidases clustering together.

Figure 4 shows the β-amino proprionitrile (βAPN) inhibition of amine oxidase activity of LOX and LOXL. LOX and LOXL were isolated from extracts of calf aorta by DEAE-cellulose chromatography. Eluates containing both immunoreactive LOX and

LOXL proteins were shown by Western blotting to lack any immuno-crossreactivity with each other. The separated fractions were then assayed for lysyl oxidase activity using an ultra-filtration assay that measures the release of ^{3}H-H_2O from an elastin substrate labeled with L-[4-5-^{3}H]lysine.

Figure 5 collectively shows the predicted folding of β-strands in the extracellular domains of cytokine receptors (Bazan, Proc. Natl. Acad. Sci. 87: 6934-6938, 1990). Fig. 5A shows a topology map with relative positions of conserved amino acids in class I (open circles) and class II (diamonds) cytokine receptors. Residues conserved in both classes are in squares. Residues fitting Prosite pattern PS00241 and conserved in LOX proteins are in darkened circles. Fig. 5B shows the predicted configuration of cytokine binding. Linked β-strands form a barrel shaped structure. The C-terminal WSxWS motif, situated between the barrels and shown in Fig. 5B, creates a pocket for cooperative binding of cytokine with a secondary binding molecule.

Figure 6 shows the amino acid alignment of the C-terminal region of human LOX and LOX-like proteins. Conserved amino acids in each protein are shaded. Residues identified with an arrow are important for predicted secondary and tertiary structural folds within cytokine receptor proteins. The putative catalytic domain and sequence with cytokine receptor homology are boxed. The tyrosine residue within the catalytic domain that is involved in the covalent linkage to lysyltyrosine quinone, is highlighted. The arrows below the sequence correspond to sequence regions predicted to form β-strands in LOX, in LOXL, in LOXL2, in LOXL3 and in LOXL4 according to the Network Protein Sequence Analysis program (http://pbil.ibcp.fr/). The position of the C-terminal amino acid residue in the sequence is indicated on the right of each peptide.

Figure 7 shows the relative abundance of LOXL3 mRNA in several different tissues, illustrated as a densitometric ratio of LOXL3 mRNA and the 2.0kb β-actin mRNA. Densitometry of autoradiograms was carried out using an AMBIS 4000 scanner.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, there are provided substantially purified lysyl oxidase-like polypeptides and functional fragments thereof. There is a striking conservation in amino acid sequence within the carboxy-terminal end of LOX and LOXL, which provides a functional similarity between these two proteins (Kim *et al.* J. Biol. Chem. 270: 7176, 1995). This is true for the copper-binding domain (containing 4 histidines), two metal-binding domains, a cytokine receptor domain, ten cysteines and the catalytic domain of lysyl oxidase. It is very likely therefore that LOXL shares a functional similarity with the copper-dependent oxidative deamination activity characteristic of lysyl oxidase.

The discovery of LOXL and additional lysyl oxidase-like proteins prompts reconsideration of the definition of a lysyl oxidase. What are the minimum requirements for a novel protein to be classified as part of the family of lysyl oxidase or lysyl oxidase-like protein? A database search for homology to conserved LOX and LOXL copper-binding domains reveals that none of the other copper-binding proteins contain this particular consensus sequence (Gacheru *et al.* J. Biol. Chem. 263: 16704-16708, 1988, and 265: 19022-19027, 1990; Krebs *et al.* Biochem. Biophys. Acta 1202: 7-12, 1993; Kosonen *et al.* Biochem. J. 32: 2283-289, 1997).

Other copper-binding proteins have distinctly different binding domains for copper. Four histidines in the copper binding domains of lysyl oxidases are critical for the binding of copper. In contrast, within the HAHl intracellular copper chaperone (Hung *et al.* J. Biol. Chem. 273: 1749-1751, 1998) in the copper-transporting ATPase important in Menkes disease, two cysteines are the critical amino acids for the formation of a co-ordinate copper complex (Vulpe *et al.* Nat. Genet. 3: 7-13, 1993) while in tyrosinase, three histidines form a complex with a copper atom (Oetting *et al.* Pigment Cell Res. 5: 274-278, 1992).

Similarly, the catalytic domain is also highly conserved within lysyl oxidases in all species studied so far (human, mouse, rat and chicken; Kim *et al.* J. Biol. Chem. 270: 7176, 1995). This domain is also present in both human and mouse LOXL but is not detected in any other known proteins. In contrast, the less defined metal-binding domains and the cytokine-binding domain, present in several lysyl oxidases, share homology with proteins unrelated to lysyl oxidases. These results indicate that LOXL is indeed a protein with a possible amine oxidase activity characteristic of lysyl oxidase.

Therefore, as used herein, lysyl oxidases or lysyl oxidase-like proteins (also can be defined as lysyl oxidase-related proteins) are defined as the proteins that contain the copper binding domain WEWHSCHQHYHSM (SEQ ID NO:11) with four histidines and the catalytic domain DIDCQWWIDITDVXPGNY (SEQ ID NO:12) containing a critical Tyr (Y) that is part of the covalently-bound carbonyl cofactor (Janes *et al.* Biochemistry 31: 12147, 1992; Krebs *et al.* Biochem. Biophys. Acta 1202: 7-12, 1993; Smith-Mungo and Kagan, Matrix Biol. 16: 387-398, 1998). A comparison of these domains within different lysyl oxidases is presented in Figure 1.

As used herein, the abbreviations of LOX refer to a polypeptide or polynucleotide of lysyl oxidase; LOXL referes to a first lysyl oxidase-like polypeptide or polynucleotide; LOXL2 refers to a second lysyl oxidase-like polypeptide or polynucleotide; LOXL3 refers to a third lysyl oxidase-like polypeptide or polynucleotide; LOXL4 refers to a fourth lysyl oxidase-like polypeptide or polynucleotide, and so on. The term "LOX-like polypeptides, polynucleotides or gene" used herein is an abbreviation refers to all the lysyl oxidase-like polypeptides, polynucleotides or gene including but not limited to LOXL, LOXL2, LOXL3 and LOXL4.

As used herein, the term "polypeptide(s)" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds. "Polypeptide(s)" refers to both short chains, commonly referred to as peptides, oligopeptides and oligomers and to longer chains generally referred to as

proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptide(s)" include those modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation, selenoylation, sulfation and transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. See, for instance: PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993; Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, Meth. Enzymol. 182: 626-646, 1990; and Rattan *et al.*, Protein Synthesis: Posttranslational Modifications and Aging, Ann. N.Y. Acad. Sci. 663: 48-62, 1992. Polypeptides may be branched or cyclic, with or without branching. Cyclic, branched and branched circular polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

As used herein, the term "functional fragment", when used in reference to a polypeptide, is a variant polypeptide having an amino acid sequence that is entirely the same as part but not all of the amino acid sequence of lysyl oxidase or lysyl oxidase-like polypeptide according to the invention. As with LOXL3 or LOXL4 polypeptides, fragments may be "free-standing" ("consisting of"), or comprised within a larger polypeptide of which they form a part or region, most preferably as a single continuous region in a single larger polypeptide.

The terms "isolated", and "substantially purified" polypeptides, may be located in, grown in, screened in, and selected from libraries using biochemical techniques familiar in the art. Such polypeptides may be natural, synthetic or chimeric and may be extracted using any of a variety of methods, such as antibody immunoprecipitation, other "tagging" techniques, conventional chromatography and/or electrophoretic methods.

As used herein, the term "substantially the same amino acid sequence" refers to amino acid sequence having at least about 70% identity with respect to the reference sequences, and retaining comparable functional and biological properties characteristic of the polypeptide defined by the reference sequence. Preferably, polypeptides having "substantially the same amino acid sequence" will have at least about 80%, more prefereably 90% amino acid identity with resect to the reference amino acid sequence; with greater than about 95% amino acid sequence identity being especially preferred.

In accordance with another aspect of the present invention, there are provided isolated lysyl oxidase-like polynucleotides. As used herein, the term "polynucleotide(s)" generally refers to any polyribonucleotide or poly-deoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotide(s)" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more

typically, double-stranded, or triple-stranded regions, or a mixture of single- and double-stranded regions. In addition, "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term "polynucleotide(s)" also includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotide(s)" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are also embraced by the term polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "polynucleotide(s)" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including, for example, simple and complex cells. "Polynucleotide(s)" also embraces short polynucleotides often referred to as oligonucleotide(s). Polynucleotides can also be DNA and RNA chimeras.

The term "isolated", when used in reference to a nucleic acid, means that a naturally occurring sequence has been removed from its normal cellular (*e.g.*, chromosomal) environment. The sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only nucleotide chain present, but that it is essentially free (about 90-95% pure at least) of non-nucleotide material naturally associated with it, and thus is distinguished from isolated chromosomes. The term "isolated" when used in reference to a nucleic acid also embraces a nucleic acid molecule being synthesized in a non-natural environment (*e.g.*, artificially synthesized). As a result, isolated nucleic acids can be produced in large

quantities and are useful in ways that natually occurring nucleic acids are not, such as identification of selective drugs or compounds.

As used herein, the term "complementary" when used in reference to a given polynucleotide sequence refers to a sequence of nucleotides which can form a double-stranded heteroduplex in which every nucleotide in the sequence of nucleotides is base-paired by hydrogen bonding to a nucleotide opposite it in the heteroduplex with the given polynucleotide sequence. The term may refer to a DNA or an RNA sequence that is the complement of another RNA or DNA sequence.

As used herein, the terms "hybridizes" and "hybridization" refer to the formation of a hydrogen-bonded heteroduplex between two nucleic acid molecules. Generally, a given nucleic acid molecule will hybridize with its complement, or with a molecule that is sufficiently complementary to the given molecule to permit formation of a hydrogen-bonded heteroduplex between the two molecules.

As used herein, the term "stringent conditions" refers to conditions that permit target nucleic acid to bind a complementary nucleic acid that has about 60%, preferably about 75%, more prefereably about 85%, identity to the target nucleic acid; with greater than about 90% identity to target nucleic acid being especially preferred. Stringency levels suitable for use to hybridize a given probe with target nucleic acid can be readily determined by those of skill in the art.

As used herein, the term "probe" refers to a polynucleotide of at least 15 nucleotides (nt), 20 nt, 30 nt, 40 nt, 50 nt, 75 nt, 100 nt, 200 nt, 500 nt, 1000 nt, and even up to 5000 to 10,000 nt in length.

In accordance with yet another aspect of the present invention, there are provided antibodies against LOX-like polypeptides. As used herein, the term "antibody" is meant to encompass constructions using the binding (variable) region of such an antibody, and other antibody modifications. Thus, an antibody useful in the invention may comprise a

whole antibody, an antibody fragment, a polyfunctional antibody aggregate, or in general a substance comprising one or more specific binding sites from an antibody. The antibody fragment may be a fragment such as an Fv, Fab or $F(ab')_2$ fragment or a derivative thereof, such as a single chain Fv fragment. The antibody or antibody fragment may be non-recombinant, recombinant or humanized. The antibody may be of an immunoglobulin isotype, e.g., IgG, IgM, and so forth. In addition, an aggregate, polymer, derivative and conjugate of an immunoglobulin or a fragment thereof can be used where appropriate. Neutralizing antibodies are especially useful according to the invention for diagnostics, therapeutics and methods of drug screening and drug design.

As used herein, the term "antigenically equivalent derivative(s)" encompasses a polypeptide, polynucleotide, or the equivalent of either which will be specifically recognized by certain antibodies which, when raised against the protein, polypeptide or polynucleotide according to the invention, interferes with the immediate physical interaction between pathogen and mammalian host.

As used herein, the term "immunologically equivalent derivative(s)" encompasses a polypeptide, polynucleotide, or the equivalent of either which when used in a suitable formulation to raise antibodies in a vertebrate, results in antibodies that act to interfere with the immediate physical interaction between pathogen and mammalian host.

As used herein, the term "immunospecific" refers to that characteristic of an antibody whereby it possesses substantially greater affinity for the polypeptides of the invention or the polynucleotides of the invention than its affinity for other related polypeptides or polynucleotides respectively, particularly those polypeptides and polynucleotides in the prior art.

In accordance with yet another aspect of the present invention, there are provided methods for producing LOX-like polypeptides by culturing a host cell containing LOX-like polynucleotide under conditions suitable for the expression of the polypeptide. As used herein, the term "host cell(s)" are cells containing endogenous polynucleotide, or

cells which have been transformed or transfected, or are capable of transformation or transfection by an exogenous polynucleotide sequence.

As used herein, the term "expression" refers to the process by which sequences encoding polypeptides or portions thereof, or polynucleotides, are produced by the host cells, or introduced or transformed into a host cell or host cell lysate by vectors containing sequences of interests for the production of the polynucleotides and polypeptides. As used herein, the term "vector" including prokaryotic or eukayotic vectors is well-known in the art. Particularly preferred vectors for transfection of mammalian cells are cytomegalovirus (CMV) promoter-based vectors and SV40 promoter-based vectors, and the like. In addition, methods of transforming or transfecting suitable host cells, as well as methods appliable for culturing said cells containing a gene encoding a heterologous proteins, are generally known in the art.

In accordance with yet another aspect of the present invention, there are provided transgenic knockout mice and methods for producing the same. Transgenic knockout mice encompassing expression of a selectable marker sequences, are animal models used for study mechanisms related to disease(s) and disorder(s). As used herein, the term "expression of a selectable marker sequence" means a detectable level expression of a selectable marker. A "detectable level" is that level of expression that can be differentiated from background expression occurring in the substantial absence of one or more factors or conditions necessary for marker expression. Detectable levels will vary depending upon the nature of the selectable marker, but will generally consist of levels at least about 10% or more greater than the background level of a selectable marker.

As used herein, the terms "disease(s)" and "disorder(s)" mean any disease or pathological conditions caused by or related to LOX or LOX-like polypeptides or polynucleotides, including, for example, a cell proliferation disorder, a cell migration disorder or a disorder related to extracellular matrix materials. The disorders contemplated herein, can be, for example, a skin disorder, a vascular system disorder, a

skeletal development disorder, a neurological disorder, a hepatic system disorder, a copper-related disorder, a pulmonary disorder, cancer, a lathyrism disorder, and the like.

Transgenic mice produced by the present invention method contain a disruption of genes related to LOX or LOX-like polypeptides in their genome. The disruption includes an insert or deletion of a transgene containing a selectable marker sequence, which can result in increase or decrease in the expression of the transgene. The disruption can also include a point-mutation of the transgene. As used herein, the term "increase or decrease in the expression" refers to a increase or decrease in the expression of a selectable marker under one set of conditions relative to the expression under another set of reference conditions. The expression of a selectable marker is increased if it is at least 10% higher than the level under reference conditions, and preferably 20%, 40%, 50%, 75%, 90%, 95% or even as much as 100% higher. The expression of a selectable marker is decreased if it is at least 10% lower than the level under reference conditions, and preferably 20%, 40%, 50%, 75%, 90%, 95% or even as much as 100% lower (*i.e.*, not expressed).

In accordance with yet another aspect of the present invention, there are provided methods for identifying compound(s) that modulate LOX or LOX-like polypeptide activity. A "compound" as used herein, is any compound with a potential to modulate the expression or activity of a lysyl oxidase or lysyl oxidase-like polypeptide. Such compounds can be LOX and/or LOX-like polypeptide specific agonist(s), antagist(s), inhibitor(s), or the like. As used herein, the term "agonist(s)" refer to agent(s) or compound(s) that enhance or increase the activity of a LOX or LOX-like polypeptide or polynucleotide. An agonist may be directly active on a LOX or LOX-like polypeptide or polynucleotide, or it may be active on one or more constituents in a pathway that leads to enhanced or increased activity of a LOX or LOX-like polypeptide or polynucleotide. On the other hand, the term "antagonist(s)" refer to agent(s) or compound(s) that reduce or decrease the activity of a LOX or LOX-like polypeptide or polynucleotide. An antagonist may be directly active on a LOX or LOX-like polypeptide or polynucleotide, or it may be

active on one or more constituents in a pathway that leads to reduced or decreased activity of a LOX or LOX-like polypeptide or polynucleotide.

Moreover, the terms "inhibitor(s)" refer to agent(s) or compound(s) that reduce or eliminate the activity or function of a LOX or LOX-like polypeptide or polynucleotide. Such reduction or elimination in activity or function also called "inhibition" or "inhibitory" effect can, for example, be in connection with a cellular component (*e.g.*, an enzyme), or in connection with a cellular process (*e.g.*, synthesis of a particular protein), or in connection with an overall process of a cell (*e.g.*, cell growth). From a molecular standpoint, such inhition effect may equate with a reduction in the level of, or elimination of, the transcription and/or translation of a specific target(s), or reduction or elimination of acvitity of a particular target biomolecule. In the present invention, such inhibition may equate with a reduction in the level of, or elimination of, the cellular effects/responses, such as amine oxidase activity, mediated by LOX or LOX-like polypeptides or polynucleotides. Such inhibition also equates with a reduction in the level of, or elimination of, the expression of LOX or LOX-like polypeptides or polynucleotides.

As used herein, the term "LOX or LOX-like polypeptide activity" encompasses all the physiological and/or phathological effects, or responses, mediated by lysyl oxidase polypeptides or lysyl oxidase-like polypeptides. Such effects or responses include, but are not limited to, interaction with extracellular matrix materials, mediation of a cell migration, or proliferation, and metabolism of intracellular, extracellular or membrane substrates, and the like.

As used herein, the term "conditions sufficient to permit said compound to interact", when used in reference to a lysyl oxidase or lysyl oxidase-like polypeptide and a compound means that the two entities are placed together, whether both in solution or with one immobilized or restricted in some way and the other in solution, wherein the parameters (*e.g.*, salt, detergent, protein or compound concentration, temperature, and

redox potential, among others) of the solution are such that the LOX or LOX-like polypeptide and the compound may physically associate.

In accordance with yet another aspect of the present invention, there are provided methods for treating a disorder associated with abnormal LOX or LOX-like polypeptide activity. The abnormal LOX or LOX-like polypeptide activity means increase or decrease in activity. As used herein, the term "increase in activity" refers to an enhanced level of measurable activity of a polypeptide relative to the measurable level of activity in a control. Activity is considered increased according to the invention if it is at least 10% greater, 20% greater, 50% greater, 75% greater, 100% greater or more, up to 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold or more than in a control.

As used herein, the term "decrease in activity" refers to a reduced level of measurable activity of a polypeptide relative to the measurable level of activity in a control. Activity is considered decreased according to the invention if it is at least 10% less, preferably 15% less, 20% less, 50% less, 75% less, or even 100% less (*i.e.*, no activity) than that observed in a control.

The present invention method of treating a subject having disorder(s) related to abnormal level of LOX or LOX-like polypeptide activity comprises administering the subject a therapeutically effective amount of a compound that modulates the LOX or LOX-like polypeptide activity. As used herein, the term "therapeutically effective amount of a compound" indicates an amount of an agent which has a therapeutic effect. Further, as used herein, a therapeutically effective amount means an amount of an agent that produces the desired therapeutic effect as judged by clinical trial results and/or animal models. This amount can be routinely determined by one skilled in the art and will vary depending on several factors.

In accordance with yet another aspect of the present invention, there are provided methods of diagnosis of a subject having or at risk of having deseases or disorders related to abnormal levels or activities of LOX or LOX-like polypeptides. The method comprises

detecting a difference in levels or activities of LOX or LOX-like polypeptide in a suspected subject relative to a normal subject. The difference is an indication of LOX or LOX-like polypeptide related desease(s) or disorder(s), which are defined above. As used herein, the term "difference" means that the levels or the activities, or both, are lower or higher in a suspected subject than those in a normal subject.

In one embodiment of the present invention, it is demonstrated that four or more different LOX-like genes: LOXL, LOXL2, LOXL3 and LOXL4 exist. In accordance of the invention, experiments have been designed to find cDNA and genomic sequences that encode regions homologous to the LOX copper binding and catalytic domains. A database protein homology search (Human Genome Sciences Inc.) identified three human cDNA clones, one of which had homology to lysyl oxidase. Using PCR primers derived from this cDNA sequence, 16 additional cDNAs were isolated from a spleen cDNA library and two PAC recombinants containing overlapping human genomic DNA, were also obtained. These clones were used to further characterize the putative lysyl oxidase-like protein. A full length cDNA sequence was established by DNA sequence analysis of several overlapping cDNA clones. An open reading frame of 3198 bp was identified that encoded the conserved copper-binding region, the catalytic domain and carbonyl cofactor and cytokine binding domains previously identified in LOX and LOXL (Jourdan-Le Saux *et al.* J. Biol. Chem. 274: (18) 12939-12944, 1999). This cDNA encodes another lysyl oxidase protein that was called LOXL2 (Figure 1).

An EST (expressed sequence tag) database (http://www.ncbi.nlm.nih.gov/dbEST/index.html) was screened for sequence homology to both the highly conserved copper binding site and the catalytic domains observed in LOX, LOXL and LOXL2. More than 1,000 lysyl oxidase-homologous EST entries were identified, 500 of these were cDNAs obtained from brain tissue libraries. A BLAST search of these EST sequences identified 21 unique and novel cDNAs. Several of these human cDNA clones were sequenced. Two of them clearly corresponded to LOXL2 cDNAs. In addition, five EST cDNAs contained overlapping sequences and were

obtained from a mixed cDNA library of fetal heart, pregnant uterus and melanocytes and cDNA libraries derived from prostate and brain mRNA. Sequence analysis of these cDNAs identified a copper binding site and the characteristic catalytic domain of lysyl oxidase. Full length cDNA sequence was generated from sequenced 5'-end clones that were isolated from brain and placenta cDNA libraries. These cDNAs encode a novel lysyl oxidase variant that is referred to as LOXL3, which represents the fourth member of the lysyl oxidase family (Figure 1). A brain specific EST search yielded a further additional member of the lysyl oxidase family, known as LOXL4. Sequence analysis of the cDNA of LOXL4 reveals a copper binding site and high level of homology to the catalytic domain common to lysyl oxidases.

Additional, non-overlapping cDNAs that were identified from brain cDNA libraries, encoded the characteristic conserved copper-binding and catalytic domains, indicating that other members of the LOX family exist.

In another embodiment of the present invention, the structures and the chromosomal locations of LOXL2, LOXL3 and LOXL4 have been identified. A thorough analysis of two overlapping PAC recombinants containing the LOXL2 gene identified a gene of 11 exons encoding the complete LOXL2 mRNA (Jourdan-Le Saux *et al.* J. Biol. Chem. 274(18) 12939-12944, 1999). Five of these exons encode the conserved copper-binding, carbonyl cofactor and cytokine binding sites shared by the LOX and LOXL genes. One PAC recombinant contained the complete LOXL3 gene having 14 extrons and 13 introns. Only three of the fourteen LOXL exons encode the conserved domains and there is significant sequence and structure divergence relative to other members of the LOX family within the eight 5'-end exons of this gene (Figure 2). The chromosomal localizations of several LOX genes are as follows: LOX maps to chromosome 5q23, LOXL maps to chromosome 15q23, LOXL2 maps to chromosome 8p21 (Jourdan-Le Saux *et al.* Genomics 2: 305-307, 1998; Jang *et al.* Genome Res. 9: 53-61, 1999), LOXL3 maps to chromosome 2p13 and LOXL4 maps to chromosome 10q24. Loss of these chromosome regions may contribute to malignant progression, such as loss

of 10q24 region may contribute to muscle-invasive bladder cancers and high grade transitional cell carcinomas of the bladder and upper urinary tract (Cappellen *et al.* Oncogene 14: 3059-3066, 1997), as well as advanced tumor stage in prostatic cancer progression (Alera *et al.* Lab Invest, 80L: 931-942, 2000).

The exon-intron structure of the LOX and LOXL genes (Boyd *et al.* Mol. Biol. Rep. 21: 95-103, 1995; Kim *et al.* J. Biol. Chem. 270: 7176, 1995) is illustrated in Figure 2 with the structure of the LOXL2 and LOXL3 genes that were determined by PCR, long range PCR and DNA sequence analysis of overlapping PAC clones obtained from Genome Systems, Inc (Jourdan-Le Saux *et al.* J. Bio. Chem. 274 (18): 12939-12944, 1999). These PAC recombinants were identified using LOXL2 and LOXL3 specific PCR generated probes. Exon-specific primers were used to PCR amplify both exon and intron sequences. DNA sequence analysis of these PCR products facilitated the design of intron-specific primers that permitted the sequence identification of all exon intron boundaries, complete exon sequences and intron sequence surrounding each exon. Intron sizes were determined from either complete sequencing or deduced from the length of PCR products using primers derived from flanking exons. Exons encoding conserved domains comprises: LOX: exons 2, 3, 4, 5 and 6; LOXL: exons 2, 3, 4, 5 and 6; LOXL2: exons 6, 7, 8, 9 and 10; LOXL3: exons 9, 10 and 11; LOXL4: exons 9, 10, 11, and 12; exons encoding nonconserved domains comprises: LOX: exon 1, LOXL: exon 1; LOXL2: exons 1, 2, 3, 4 and 5; LOXL3: exons 1, 2, 3, 4, 5, 6, 7, 8 and 12; LOXL4: exons 1, 2, 3, 4, 5, 6, 7 and 8; and the exons containing the 3'UTR comprises: LOX: exon 7; LOXL: exon 7; LOXL2: exon 11; LOXL3: exon 13; LOXL4: exon 13. No significant conservation of sequence within the 3'UTR was noted among the genes.

cDNA sequences encoding LOXL2 (SEQ ID NO:5), LOXL3 (SEQ ID NO:7), and LOXL4 (partial; SEQ ID NO:9) and the predicted amino acid sequences LOXL2 (SEQ ID NO:6); LOXL3 (SEQ ID NO:8) and LOXL4 (partial sequence; SEQ ID NO:10) are presented herein.

In another embodiment of the present invention, the molecular structure of LOX and LOX-like proteins are determined. Based upon sequence and gene structure similarities, there seems to be a close evolutionary relationship between the LOX and LOXL genes and the LOXL2 and LOXL3 genes. It is possible that at least part of the LOX, LOXL, LOXL2 and LOXL3 genes share a common ancestor as the exons encoding the functional domains of the mature protein remain closely homologous in sequence and size. In contrast, exons encoding the 5' and 3' untranslated regions and the amino-terminal coding domains of these proteins have diverged significantly in sequence and size (Figure 3). LOXL4 also appears to share a close evolutionary relationship with other LOXL genes, particularly LOXL3, based on sequence similarity.

The evolutionary distance among various members of the lysyl oxidase family reveal significant structural and functional relationships. There is significant evolutionary divergence at the amino-terminal end of the LOX proteins. The potential nuclear localization signal described by Kagan and co-workers (Li *et al*. Proc. Natl. Acad. Sci. USA 94: 12817-12822, 1997) is present in LOX, LOXL and LOXL3 but not in LOXL2, providing that although LOXL2 can have an intracellular location, it appears to be transported by a different mechanism.

In another embodiment of the present invention, the catalytic activities of the invention proteins are tested. According to the molecular structures of the invention proteins, two domains are conserved, in addition to the copper binding site. These are the catalytic domain and the cytokine/chemokine binding domain. While the catalytic function of LOX is well understood, at least within the extracellular matrix, the role of the cytokine binding domain within the LOX proteins is unknown. It has been tested whether the catalytic domain present in the LOXL proteins reflects function similar to the cross-linking activity of LOX. While the results indicate differences in substrate specificity (Kim *et al*. J. Cell Biochem. 72: 181-188, 1998), it is not clear whether the catalytic activity of LOXL is different to LOX.

To explore the possibility that LOXL will catalyze the oxidative deamination of lysyl residues, the cross-linking activity of the LOXL protein on an elastin substrate was tested. It was reported that the significant cross-linking activity for a bovine aorta-derived LOXL was detected. This activity was previously described for LOX only and was inhibited by a known and specific LOX cross-linking activity inhibitor, β-amino proprionitrile (βAPN) (Figure 4). These *in vitro* results indicate that both LOX and LOXL can catalyze the oxidative deamination of lysine residues in an elastin substrate. While this finding does not address whether LOX and LOXL specifically utilize an elastin substrate, it does indicate that all lysyl oxidases, including LOXL2, LOXL3 and LOXL4, can exhibit this βAPN inhibitable catalytic activity.

In accordance with the present invention, it has been discovered a cytokine binding domain that overlaps with the catalytic domain in the invention proteins. The C-terminal region of human LOX and LOX-like proteins contains sequence homology with the N-terminal extracellular domain of the growth factor and cytokine receptor superfamily that overlaps the catalytic site (Figure 5). The consensus sequence found in the N-terminal modules of Class I receptors, C-X_9-C-X-W-X_{26-32}-C X_{10-13}-C (SEQ ID NO:13) (where C is cysteine, W is tryptophan and X, is a defined number of any amino acid) is conserved in human LOX and LOX-like proteins (Kim *et al.* J. Biol. Chem. 270: 7176, 1995). Furthermore, the first 13 residues fit the Prosite pattern PS00241, C-[LVDYR]-X(7,8)-[STIVDN]-C-X-W (SEQ ID NO:14), found in cytokine receptors, where amino acid residues in [ ] indicate the presence of residues but not in any particular sequence and numbers in ( ) indicate the number of consecutive residues. Examples of proteins in this class include erythropoietin receptor, granulocyte colony-stimulating factor receptor, granulocyte-macrophage colony-stimulating factor receptor alpha chain, growth hormone receptor, thrombopoeitin receptor, and various interleukin receptor chains (Miyajima *et al.* AM. Rev. Immunol. 10: 295-331, 1992). Structural analysis of such receptors provides that they are related to immunoglobulin constant domains and

evolved from primitive fibronectin type III sequence modules that are also common to various adhesion molecules (Bazan, Proc. Natl. Acad. Sci. 87: 6934-6938, 1990).

Three-dimensional crystallographic analysis of the cytokine receptors provides that the N-terminal extracellular domain forms two barrel-shaped modules, each consisting of six or seven β-strands (Bazan, Proc. Natl. Acad. Sci. 87: 6934-6938 1990; see Fig. 5). Cytokines are presumed to bind into the groove created by a kink between these two modules. Based on this model the C-terminal region of human LOX and LOX-like proteins lacks sequence corresponding to the C-terminal "barrel" module. The LOX and LOX-like proteins contain cysteine and tryptophan residues at conserved positions within the first four and five β-strands that form the N-terminal barrel module (Figure 5, strands A-E).

Despite these sequence differences, secondary structure analysis predicts that β-strands are formed (Figure 6) and this region of the LOX and LOX-like proteins can fold in a similar manner. The putative catalytic domain is within this module and it is plausible that this ancient structure contributes to defining the active site.

In accordance with another aspect of the invention, it has been discovered that the catalytic site is embedded within a larger partial cytokine binding domain in LOX and LOX-like proteins. Predicted β-strand structure within this larger domain indicates similar secondary structure for LOX and LOXL that differs only in one β-strand. This difference can define a different catalytic site within these proteins and can result in different substrate specificity for LOX and LOXL. The secondary structure analysis of LOXL2 and LOXL3 predicts four β-strands in both proteins and consequently a different structure within the catalytic and cytokine domains. The presence of the partial cytokine binding site overlapping the catalytic site raises the possibility that the LOX proteins can fulfill biological roles different from the catalytic function reported for LOX and LOXL. Evidence for such roles comes from the work of Lazarus (Matrix Biol. 14: 727-731,

1994) who showed a potential role for LOX that involved the active site in leukocyte motility.

In another embodiment of the present invention, differential expression of the LOX and LOX-like genes in specific tissue and cellular localizations have been observed. An overlap in the patterns of appearance of LOX and LOXL mRNAs in different tissues has been demonstrated (Kim *et al.* J. Biol. Chem. 270: 7176, 1995). RNA from adult human heart, placenta, lung and kidney contain abundant quantities of both LOX and LOXL mRNAs. The tissue distribution of the LOXL2 mRNA is, however, strikingly different from the mRNAs encoding both LOX and LOXL. Low levels of the 3.6 kb LOXL2 mRNA were noted in heart, lung and kidney but in abundant levels in the placenta, the uterus and the prostate. Quantitative analysis of LOXL2 mRNA in 16 different human tissues relative to β-actin mRNA has recently been reported (Jourdan Le-Saux. *et al.* J. Biol. Chem. 274 (18): 12939-12944, 1999). A similar analysis of LOXL3 mRNA levels revealed steady state message at higher levels in uterus, testis, colon and spleen (Figure 7). Longer exposure of the Northern blots detected low levels of the 3.1 kb LOXL3 mRNA in placenta, prostate and heart. The Northern blot analysis of LOX and LOX-like mRNAs provides an insight into the expression profiles of the LOX and LOX-like genes in many human tissues. It also provides a complex expression pattern and a great variation in mRNA levels for each LOX-like gene in different tissues. The LOX and LOXL genes appear to be expressed in most tissues, while the expression of the LOXL2 and LOXL3 genes is more restricted. LOXL4 mRNA is expressed in the testes, smooth muscle cells and pancreas. For example a 3.5kb mRNA encoding by the LOXL4 gene is detected using Northern blot analysis in MB-231 invasive breast cancer cells, human osteosarcoma, HeLa and PC-3 metastatic prostate carcinoma cells.

In another embodiment of the present invention, it has been discovered that the LOXL2 gene is specifically expressed by trophoblasts in the placenta. Using *in situ* hybridizations, it has been established that the highest fetal level of LOXL2 is detected in placental tissue while no LOXL2 is detected in any other cells in fetal membranes. In the

placenta, positive autoradiographic signals are associated with syncytial and cytotrophoblasts which appear to be responsible for the synthesis of LOXL2 mRNA in placental tissue. These findings are consistent with an earlier description of a protein that had been identified in the placenta with LOX activity but which was, however, different from LOX (Kuivaniemi *et al.* J. Biol. Chem. 259: 6996-7002, 1984). Moreover, while LOX has been shown to be present in the amnion, one of three components that make up the fetal membranes (Casey & MacDonald, J. Clin. Endocrin. Metab. 82: 167-172, 1997), LOXL2 mRNA is not detected in fetal membranes. In contrast, LOXL2 mRNA is synthesized by trophoblasts and is present in regions of the placenta where the LOX mRNA does not appear. These *in situ* hybridization data provide that LOXL2 mRNA appears at non-overlapping locations with LOX mRNA and is localized to the placental cytotrophoblasts that lay down a basement membrane. The trophoblastic basement membrane separates these cells from the villous stroma. Localization of LOXL2 mRNA with basement membrane synthesizing cells provides the possibility that LOXL2 can be associated with basement membrane. Subsequent immuno-histology using LOXL2 antibody on skin and brain sections confirmed such an association.

In accordance with another embodiment of the present invention, LOX and LOXL2 have been detected in different areas of the skin. Immunohistochemistry of mouse skin using LOX and LOXL2 specific antibodies shows general distribution of the LOX proteins in regions of the epidermis where fibrillar collagens and elastin are known to be deposited. In contrast, LOXL2 is detected in areas where LOX does not appear. These areas are around cells that deposit basement membrane, the epithelial cells of the dermis, at the outer sheath layer of the hair follicles that is continuous with the epithelial cell layer of the dermis, and around the skeletal muscle cells.

LOX and LOXL specific antibodies have been used in several studies (Decitre *et al.* Lab. Invest. 78: 143-151, 1998). The design and generation of a LOXL2 and/or other LOX-like specific antibodies are described in detail hereafter (see Example 4 below).

In accordance with another embodiment of the present invention, it has also been discovered that LOX, LOXL and LOXL2 are present in different regions of the developing mouse brain. It has been reported that LOX and LOXL2 mRNAs are not co-incident in the same cells in the placenta. Moreover, the LOX and LOXL2 proteins are located within different regions of mouse skin. The independent location of LOX and LOXL2 provides different functions for these two proteins. To further explore the spatial separation of LOX and LOXL2 the distribution of LOX and LOXL2 in the developing mouse brain is also analyzed.

In accordance with the invention, it has been possible to identify temporally and spatially divergent expression for some of the LOX genes during embryonic development. Immunohistochemistry of 9 and 15 day old mouse embryos using LOX, LOXL and LOXL2-specific antibodies, detects abundant amounts of all three proteins in the heart, lung and around the neural tube of the developing embryo. Even though the expression of the LOX and LOXL genes in brain as detected by Northern analysis is low, the distribution of each LOX protein is strikingly different. The LOXL2 protein is detected in areas in the brain of the 15 day old mouse embryos limited to the ependymal cell layer lining of the ventricles, the small blood vessels within the choroid plexus and cerebrum and the mesenchymal cells surrounding the neural tube (destined to form the meningeal membranes). These connective tissue areas also stain with a known extracellular matrix protein, laminin (Thomas and Dziadek, Exp. Cell Res. 20: 854-67, 1993; Villanova *et al.* J. Submicrosc. Cytol. Pathol. 29: 409-413, 1997). Since the mesenchymal cells are of neuroepithelial origin, the detection of LOXL2 in this cell layer is in good agreement with the identification of one of the LOXL2 ESTs in a neuroepithelial cell cDNA library. LOX, in contrast, is found only at three distinct locations: in the secretory surface of ependymal cells of the choroid plexus, within the cerebellar folia and deep cerebellar nuclei associated with neurofibers, and within the molecular layer of the dentate gyrus of the hippocampus.

Observations from Northern analysis of tissues of the central nervous system indicate that LOXL3 is expressed at yet other distinct areas: in the frontal lobe, medulla, and spinal cord. However, the precise distribution of LOXL3 within these brain sections can only be assessed once the immuno-localization of LOXL3 is completed.

The appearance of LOX within the extracellular matrix of the cerebellum and hippocampus, most likely synthesized by astrocytes, is consistent with the description of an extracellular localization for LOX. However, both LOX and LOXL can also detected inside neurons where these amine oxidases have the potential to act upon such substrates as dopamine and serotonine and play an important role in the function of the central nervous system. Moreover, the presence of LOXL2 around small blood vessels of the choroid plexus and blood vessels in the cerebellum coincides with the presence of epithelial cells, endothelial cells and importantly, the basement membrane that these cells synthesize and contributes to the integrity and function of the cerebrovascular system.

Therefore, in accordance with the present invention, LOX and LOXL2 have been detected in several tissues (placenta, skin, and central nervous system) in distinctly different regions, apparently synthesized by different cell types. LOX, LOXL and LOXL2 all seem to be associated with components of the extracellular matrix. LOXL2, however, is consistently associated with cells active in the assembly of basement membrane.

In another embodiment of the present invention, a comparison of the hydropathy plots for LOX, LOXL, LOXL2, LOXL3 and LOXL4 confirms the presence of the signal peptide at the amino terminus of all LOX-like proteins (Trackman *et al.* J. Biol. Chem. 267: 8666-8671, 1992; Kagan *et al.* J. Cell. Biochem. 58: 1-10, 1995; Li *et al.* Proc. Natl. Acad. Sci. USA 94: 12817-12822, 1997). A more detailed immuno-histologic analysis of LOX, LOXL and LOXL2 localization in human dermal fibroblasts in culture, provides secretion of LOX into the extracellular matrix and localizes the LOXL2 protein associated with the cell membrane. The LOX and LOXL2 immuno-staining of dermal

fibroblasts reveals LOX and LOXL2 within the transport vesicles, within channels of the endoplasmic reticulum and a plasma membrane localization for LOXL2 while LOX and LOXL are not detected associated with the cell membrane. All the LOX-like proteins are also detected within the nuclei of cultured cells to varying degrees.

To further explore the observed membrane-associated localization of LOXL2, the LOXL2 amino acid sequence has been analyzed. Two sequence motifs that are known to be present in the extra-cellular domains common in trans-membrane cell surface receptors are detected at the amino terminal end of the protein as well as within LOXL3 and LOXL4. Moreover, these sequence motifs, the Scavenger Receptor Cysteine Rich (SRCR) motifs, are absent in LOX and LOXL.

Both LOXL2 and LOXL3 have all the critical amino acids within the long extracellular SRCR consensus sequence motif GAY IGE GRV EVL KNG EWG TVC DDK WDL VSA SVV GRE LG (SEQ ID NO:15). For LOXL2, this sequence is between amino acids 130-167 and for LOXL3 between amino acids 312-349. There is a similarly strong homology to the short extracellular SRCR motif CSHSQDAGVRC (SEQ ID NO:16), both in LOXL2, LOXL3 and LOXL4. These sequence motifs are only present in cell surface receptors such as T-cell differentiation antigen, CD-6 precursor, macrophage scavenger receptor, MSRE and the speract receptor, SPER (Aruffo *et al.* J. Exp. Med. 174: 949-952, 1991).

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLE 1

Isolation of a cDNA encoding human LOXL3 and LOXL4

The mouse *lor-2* cDNA sequence (AF053368, Jang *et al.* Genomics 2: 305-307, 1999) was used to search a human EST database by the BLASTN search program (http://www.ncbi.nlm.nih.gov/cgi_bin/BLAST). Three overlapping ESTs (AI752772,

AA852888 and R55706) in Genbank database having significant homology to the *lor-2* cDNA were identified. Complete DNA sequencing of this contig was performed using ABI BigDye terminator cycle sequence with an ABI310 automated DNA sequencer as described by the manufacturer. The assembled contig (submitted to GenBank under accession number AF311313) has 3121 nucleotides, containing an open reading frame (ORF) of 2262 nucleotides encoding a putative polypeptide of 754 amino acids with a predicted molecular mass of 83 kD. Within the 787 base long 3'-untranslated sequence three AU-rich sequence elements are present, which are usually found within the 3' trailer region of unstable mRNAs (Lewin, Genes, Oxford University Press, Oxford, 1997). The conserved C-terminal region shows 62% nucleic acid identity to LOX. The deduced amino acid sequence contains a signal peptide sequence indicating the extracellular location of this protein. The five predicted N-glycosylation sites are found throughout the protein at amino acid positions 111, 266, 339, 481 and 625. In contrast, the N-glycosylation sites in LOX at amino acid positions 81, 97 and 144 are all within the pro-peptide domain and are not present within the active LOX.

The putative polypeptide encoded by the cDNA contig presents all structural characteristics of the LOX enzyme family. The copper-binding site with 4 histidyl residues (WVW**HECHGHYHS**M (SEQ ID NO:17) is at amino acid positions 601-612. Though all four histidyl residues are conserved, three amino acid differences are noted from the LOX and LOXL copper-binding sites (W**E**WHSC**HQ**HYHSM (SEQ ID NO:11) (Figure 1). Lysyl (Lys^{639}) and tyrosyl (Tyr^{670}) residues corresponding to residues of the lysyl tyrosyl-quinone co-factor (Lys^{314} and Tyr^{349}) are also present (Figure 1). The C-terminal region displays significant sequence homology to the extracellular domain of members of the growth factor and cytokine receptor superfamily. This cytokine receptor-like motif (residues 666-727) overlaps with the conserved active site as in the LOX, LOXL and LOXL2 proteins (Kim *et al.* J. Biol. Chem. 270: 7176, 1995). Moreover, four Scavenger Receptor Cystein-Rich (SRCR) domains (Resnick *et al.* TIBS 19: 5-8, 1994) are also found in the N-terminal region of the protein using the MOTIF software

(hppt://www.motif.genome.ad.jp) at amino acid positions 44-144, 186-281, 307-407 and 417-526 (Figure 1). Based on these characteristics with members of the LOX family, this cDNA is refered to as a human LOXL3.

The procollagen C-proteinase/BMP-1 cleavage site, GDD, described with LOX (Panchenko *et al.* J. Biol. Chem. 271: 7113-7119, 1996), is also found at amino acid position 446-448 of the LOXL3 protein. If proteolytic processing of LOXL3 occurs at this position, the predicted size of the cleaved product would be 306 amino acids in length with a molecular mass of 35 kD. This putative BMP-1-processed LOXL3 would not contain any of the SRCR motifs. However, the copper binding-site, the lysyl and tyrosyl residues, and the conserved sequence motifs surrounding these sites, will be retained in this cleaved form and can confer copper-dependent amine oxidase activity to LOXL3.

A brain specific EST search yielded an additional clone. Sequence analysis of the cDNA reveals a copper binding site and high level of homology to the catalytic domain common to LOX. This clone is referred to as LOXL4. The full length coding sequence of LOXL4 was amplified from placental RNA. The sequence was confirmed using a chromosome 10 genomic clone that was also used to determine the gene structure of LOXL4 and assign the gene locus to 10q24.

EXAMPLE 2

Chromosome assignment and genomic organization of LOXL3 and LOXL4

A full-length LOXL3 cDNA sequence was used to search the non-repetitive human genomic sequence database at GenBank by the BLASTN search program. Alignment analysis between the cDNA and genomic sequence was performed to determine the boundaries of exons and introns. Chromosomal localization is determined by radiation hybrid mapping. Basically, a human BAC clone bac91a19 (AC006544) was identified using the entire LOXL3 cDNA sequence. This clone has been mapped to chromosome 2p13. In addition, the LOXL3 cDNA matches with 100% identity with the

coding regions of two other overlapping human genomic sequence entries (AC005033 and AC005041) which have also been mapped to chromosome 2p13.3. Therefore, the chromosome location of the human LOXL3 gene is mapped to the 13.3 region of the short arm of chromosome 2 (Figure 2). Using the same method, LOXL4, on the other hand, is mapped to chromosome 10q24 region.

An alignment between the LOXL3 cDNA and the genomic sequences provides that the LOXL3 gene spans about 21 kb of genomic DNA and contains 14 exons and 13 introns. Three of the 14 exons encode the conserved amino oxidase domains (Figure 2). Significant sequence and structural divergences from the other LOX-like genes are found within the first eight exons of this gene.

The 3' end of the LOXL3 gene overlaps with the *Htr* A2 serine protease gene. The BLASTN analysis reveals a complete sequence identity between the 3' end of the human LOXL3 cDNA and three nuclear serine protease cDNAs (AF141305, AF141306 and AF141307) known to be involved in mammalian stress response (Gray *et al.* Eur. J. Biochem. 267: 5699-5710, 2000). These cDNAs are splice variant transcripts of the human *Htr*A2 gene, which has also been mapped to chromosome 2p13. The 3' end of the LOXL3 cDNA is found to overlap by 359 nucleotides with the *HtrA2* serine protease cDNA (AF141305), by 357 nucleotides with *HtrA2-p7* splice variant (AF141306) and by 497 nucleotides with splice variant *HtrA2-p4* (AF141307). The overlapping region includes the last 165 coding nucleotides and the 3'UTR for the *HtrA2* serine protease gene and the last 503 nucleotides of the 3'UTR of the LOXL3 gene. The LOXL4 gene has a very similar structure to LOXL3; out of the 13 exons, four encode the conserved domains.

EXAMPLE 3

Tissue specific expression of the LOXL3 and LOXL4 gene

Expression of LOXL3 and LOXL4 genes is examined by Northern blot analysis. Two Multiple Tissue Northern (MTN) blots (Clontech Laboratories, Palo Alto, CA) containing two μg aliquots of size-separated poly(A+) RNA obtained from 16 human adult tissues are used. Each MTN blot was prehybridized for 4h. at 42°C in 10 ml of Hybrisol II (Oncor, Gaithersburg, MD). Hybridization, using ^{32}P-labeled cDNAs prepared by random primer labeling, were carried out in 7.5 ml of Hybrisol II at 42°C for 12 h. After washing blots twice in 2XSSC, 0.5% SDS at room temperature and twice in 0.1XSSC, 0.1% SDS at 50°C and exposed to Kodak Biomax film for 12 h. at –70°C, the blots were stripped and reprobed with a radiolabeled β-actin cDNA. The specific activity of the radiolabeled cDNA probes used was $5x10^9$dpm/μg. The relative abundance of LOXL3 and LOXL4 mRNAs in these tissues is presented as a densitometric ratio of LOXL3 or LOXL4 mRNA and the β-actin mRNA. Densitometry of autoradiograms was carried out using an AMBIS 4000 scanner.

Northern blot analysis provides an approximately 3.1 kb LOXL3 mRNA in all tissues examined. The size of the LOXL3 mRNA determined by the Northern blot analysis is consistent with the length of the cDNA sequence (3121 nucleotides). High levels of LOXL3 expression were found in uterus, testis, colon and spleen (Figure 7). Longer exposure detected low levels of LOXL3 expression in placenta, prostate and heart. Northern blot analysis also reveals the expression of LOXL3 mRNA in neurons, leukoccytes and brain. In subsequent Northern blot analysis of sections of the central nervous system, low levels of LOXL3 mRNA were detected in almost all sections. The highest levels of expression were found in spinal cord and medulla.

LOXL4 mRNA is expressed at low levels in most tissues except testes, smooth muscle and pancreas. The expression of LOXL4 is also detected in the metastatic and

invasive breast carcinoma cell line MB-231 and in metastatic prostate carcinoma cell line PC-3, while no expression of LOXL4 is detected in the non-metastatic, non-invasive breast carcinoma cell lines MCF-7 and T47D. These results demonstrate that LOXL4, like LOX, is up-regulated in association with the metastatic phenotype described in breast cancer (Kirschmann *et al.* Breast Cancer Research and Treatment 55: 127-136, 1999).

EXAMPLE 4

Antibodies for LOX-like polypeptides, for example, antibodies specific for LOX, LOXL1, LOXL2, LOXL3 and LOXL4

Human antibodies are generated against divergent domains from LOX, LOXL and LOXL2 that do not exhibit any obvious antigenic cross-reactivity. Moreover, these antibodies all specifically react with the mouse homologues of LOX, LOXL and LOXL2 and can be used to analyze LOX, LOXL and LOXL2 localization in both human and mouse tissue.

A region of human LOXL3 and LOXL4 cDNAs, divergent between different LOXLs, but highly conserved between human and mouse can be cloned, expressed and used to generate a LOXL3 or LOXL4 specific antibody.

To generate the polyclonal LOXL3 and LOXL4 antibodies, the custom peptide and antisera service provided by Genosys are used. The derived, unique LOXL3 or LOXL4 peptide sequence is provided to Genosys and the LOXL3 or LOXL4 specific peptide synthesized and HPLC purified and then conjugated to either keyhole limpet hemocyanin or BSA, using water soluble carbodiimide. The conjugates are purified by gel filtration, freeze-dried, reconstituted in water, mixed with Freund's adjuvant and injected subcutaneously into rabbits at multiple sites. After continued injections every 14 days, the pre-immune serum and immune sera are obtained after 49, 63 and 77 days from the first injection of the conjugate.

Strategies and methods described herein are also applied for making antibodies specific for other LOX-like polypeptides.

EXAMPLE 5

Characteristics of LOX and LOXL as secreted extracellular matrix proteins having amine oxidase activities specific for certain fibrillar collagens and elastin

The experiments covered by this example include, but are not limited to 1) determination of the relative temporal and spatial localization of LOX and LOXL during the development of liver fibrosis and during aorta development and identification of cells within these tissues responsible for the synthesis of these proteins; 2) determination of co-localization of LOX and LOXL with type I and type III collagens during the development of liver fibrosis and co-localization of LOX and LOXL with adventitial collagen fibrils and elastin in the developing aorta; and 3) determination of the *in vitro* catalytic activity of LOX and LOXL using type I and type III collagen specific peptide substrates and elastin. Similar strategies and methods are also applied for evaluation of other LOX-like polypeptide.

The methods for performing these experiments are described in detail as follows. Standard techniques, such as DNA sequencing, Southern, Northern and Western blot analysis, DNA cloning, nucleic acid radiolabeling and cell culture techniques are used as described, for example in Maniatis *et al.* Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1982; Sambrook *et al.* Molecular Cloning: A Laboratory Manual (2 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989; Davis *et al.* Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, 1986 and other publications.

Tissue specimens: A mouse model for liver fibrosis is used. This model was developed in adult mice following repeated intraperitoneal injections of carbon tetrachloride (Kim *et al.* J. Cell. Biochem. 72: 181-188, 1998). To analyze LOX and

LOXL accumulation in the developing aorta, normal adult mice are bred and the aortic tissue obtained from prenatal animals at 15 days of gestation, newborn mice at 3, 6 and 12 weeks of age. It is known that elastin synthesis in the aorta is initiated in late prenatal development and terminates in early adulthood. Therefore, to analyze LOX and LOXL coincident with elastin synthesis during aortic development, it is necessary to cover these developmental ages.

Immunohistochemistry of the LOX gene products: Freshly isolated tissue sections are fixed overnight in 4% paraformaldehyde at room temperature then embedded in paraffin. Cut sections are mounted on glass slides and rehydrated by rinsing in xylene, 100% ethanol, 95% ethanol and 70% ethanol. After 30 min incubation in TBS (Tris buffered saline) and a 5 min incubation in water, the endogenous peroxidase activity is blocked with 3% hydrogen peroxide. The sections are then digested with Trypsin for 20 min at 37°C and the non-specific tissue sites blocked using 1% normal rabbit serum in TBS for 30 min and Power Block (BioGenex) for 10 min. After 2 washes in TBSBSA, slides are incubated with appropriately diluted primary antibodies overnight at 4°C. Antibody binding is detected using a biotinylated anti-rabbit immunglobulin linker and peroxidase-conjugated streptavidin label and visualized with diamobenzidine (DAB) as chromogene (BioGenex). After washing in TBS and water, the sections are counterstained with Mayer's hematoxylin.

Confocal microscopy analysis: Methods can be used basically as described. Fibroblasts are grown in subconfluent cultures and fixed in either 100% methanol for 5 min (to visualize transport vesicles) or in 3% paraformaldehyde in the presence of NP40 for 30 min (to visualize the endoplasmic reticulum), or in 100% methanol for 30 min at –20 °C (to visualize the cell surface). Following washes in PBS, cells are incubated with LOX and LOXL2 or LOXL3 or LOXL4 specific antibodies (1:100 dilution for 1 hour). A secondary goat anti-rabbit flurescein conjugated antibody is applied at 1:200 dilution for 1 hour.

In situ hybridization: Methods are used as described by Bogic *et al.*, (J. Clin. Endo. Metab. 80: 130-137, 1995) and have been successfully used to detect LOXL2 mRNA in the placenta (Jourdan-Le Saux *et al.* J. Biol. Chem. 274 (18): 12939-12944, 1999). Such methods can also be used to detect other LOX-like mRNAs.

FLAG-labeled LOX and LOXL expression constructs: Full length cDNAs for both LOX and LOXL are constructed and inserted into the expression vectors ZeoSV2 (uses an SV40 promoter) and pcDNA 3.1 (contains the CMV promoter). A PCR-based procedure can be used to add the FLAG coding sequence and a stop codon as an in-frame insertion 3' of the LOX and LOXL cDNA coding sequence and 5' of the BGH 3'UTR. Following sequence confirmation, LOXL recombinants are transiently transfected into HeLa cells and fibroblasts using lipofectamine. It has been shown that too high a level of expression of either LOX or LOXL can lead to growth arrest of transfected cells. In general, the CMV promoter can usually direct the expression of much higher levels of recombinant protein than the SV40 promoter. Therefore, these promoters can be tested to determine appropriate expression levels of LOX and LOXL protein without inhibiting, to any significant extent, the growth of transfected cells.

Immunogold labeling and electron microscopy: Indirect immunogold labeling and transmission electron microscopy can be used for the specific extracellular, intracellular and nuclear localization of the LOX proteins. Mouse tissue sections are dissected into 1 mm^3 pieces and fixed in 4% paraformaldehyde in 0.1 M Sorensen's buffer, pH 7.4 for 4-6 hrs at 4°C. After several changes of Sorensen's buffer, the tissue is placed in 20 mM Tris-HCl, pH 8.0 and treated with 6 M guanidine-hydrochloride, rinsed in Tris buffer and retreated with 100 mM iodoacetamide. The tissue is then washed in several changes of Sorensen's buffer containing 4% sucrose and left overnight at 4°C. The next day, the tissues are dehydrated in a graded series of methanol at progressively lower temperatures to –20°C and finally embedded in Lowicryl K4M at 35°C. Lowicryl blocks are polymerized by UV illumination for 24 hrs at –34°C and an additional 48 hrs at –10°C.

Lowicryl thin sections are cut and placed on formvar coated nickel grids and incubated face-down on drops of blocking solution that comprise 50 mM Tris-HCl, pH 6.0, 100 mM NaCl and 1% BSA. After 15 min, grids are transferred to drops of primary antibody diluted in blocking solution and left overnight in a humidity chamber at 4°C. After exposure to the primary antibody, the grids are washed 3 times for 10 min each, on wells containing 50 mM Tris-HCl, pH 6.0 with 100 x nM NaCl and 0.1%Tween-20. Grids are then incubated on blocking solution for 15 min prior to being transferred to drops of goat anti-rabbit IgG conjugated to 10 nm colloidal gold diluted to 1% in blocking solution. After a 1 h exposure to this secondary antibody, grids are washed as described before exposure to the primary antibody and rinsed 3 times for 5 min each in distilled water. Immunolabeled sections are counter-stained with methanolic uranyl acetate followed by lead citrate and viewed at various magnifications in a Zeiss 10/A transmission electron microscope.

Immunopurification of the LOXL proteins: The expressed LOX and LOXL proteins can be tested for activity in the media and in the cell extract. HeLa cells are lysed as described by Garell (Method. Enzymol. 100: 411-413, 1983). Media and cell lysates are applied onto immunoadsorbent columns. The immunoadsorbent is made using an anti-FLAG antibody conjugated to agarose beads as described (Decitre *et al.* Lab. Invest. 78: 143-151, 1998). To bind the expressed LOX proteins to the immobilized FLAG antibody, agarose beads are equilibrated in TBE buffer (50 mh4 Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA). The crude HeLa cell extract containing the overexpressed FLAG-tagged LOX proteins is dialyzed against the same TBE buffer and mixed with the agarose beads for 16 hrs at 4°C. The mixture can be subsequently prepared in a column and washed with TBE. The immunoadsorbed FLAG-LOXL is then eluted with a solution of 0.2 M glycine, pH 2.8 and collected in a neutralizing buffer (1 M Tris-HCl, pH 8.0).

Catalytic activity assay: The immunopurified LOX and LOXL proteins can be assayed for catalytic activity by the method described (Gacheru *et al.* J. Cell. Biochem. 65: 395-407, 1997). A recombinant tropoelastin substrate is prepared and biosynthetically

labelled with [4,5-^{3}H]-L-lysine as described (Bedell-Hogan *et al.* J. Biol. Che, 268: 10345-10350, 1993). Aliquots of the immunopurified LOX proteins are incubated for 2 h at 37°C with 125,000 cpm of the tritium-iabelled tropoelastin substrate in assays brought to a final volume of 800 µl with 100 mM sodium borate, 150 mM NaCl, pH 8.0. Tritiated water released during the incubation as a result of LOX and LOXL enzymatic activity is isolated by distillation *in vacuo* and quantified by liquid scintillation counting.

In collagen types I and III, LOX derived oxidation is restricted to the single lysine, in the N- and C- telopeptide, non-triple helical sequences. In the N-terminal telopeptides the consensus sequence is X-Asp-J-Lys-Z (SEQ ID NO:18) (X: Tyr or Phe; J: variable). To test LOX and LOXL activity towards these collagen substrates the corresponding N-terminal synthetic peptides can be synthesized and assayed by a continuous fluorescent method (Trackman *et al.* Anal. Biochem. 113: 336-342, 1981) and expressed as a ratio of Kcat/Km (min^{-1}; mM^{-1}) (Kagan *et al.* In: Molecular Biology and Pathology of Elastic Tissue, (Editors: Mecham and Roberts), Ciba Foundation Symposium Series, 1994).

Control samples can be enzyme extracts incubated in the presence of P-aminoproprionitrile. Activities can be calculated as βAPN- inhibitable counts of tritium released using this specific inhibitor of lysyl oxidase (Kagan *et al.* In: Molecular Biology and Pathology of Elastic Tissue, (Editors: Mecham RP. and Roberts L., Ciba Foundation Symposium Series, 1994). Activity measurement can be done in at least three independent experiments.

LOX and LOXL are secreted proteins deposited in the extracellular matrix and responsible for cross-linking different extracellular matrix proteins (Kim *et al.* J. Cell. Biochem 2: 181-188, 1998). A mouse model of carbon tetrachloride induced liver fibrosis has been used to demonstrate increased LOX mRNA levels coincident with increased levels of pro-alpha 1 (I) collagen mRNA, while increased LOXL mRNA levels are coincident with increased levels of pro-alpha 1 (III) collagen mRNA during the

development of liver fibrosis. It has been determined that the LOXL gene and the gene encoding pro-alpha 1 (III) collagen are coordinately regulated and moreover, can facilitate the availability of LOXL for crosslinking of type III collagen during the deposition of fibrillar collagen in the fibrotic liver. Similarly, the coordinate expression of the LOX gene and a type I collagen gene indicates that LOX is the extacellular amine oxidase specifically required for crosslinking type I collagen. The appearance of LOX and LOXL and collagen type I and III in the extracellular matrix is determined using immunostaining and confocal microscopy and immunogold labeling and electron microscopy. It has been determined that LOXL is largely associated with type III collagen fibrils and LOX associated with collagen type I fibers.

There is no significant amount of elastin deposited during the development of liver fibrosis. In order to determine if LOX and/or LOXL are also responsible for the formation of the desmosine crosslinks in the developing elastic fiber, LOX and LOXL are studied in the developing mouse aorta. It is known that elastin synthesis in the developing aortic wall is largely confined to the smooth muscle cells of the media. The outer adventitial layer of the arterial wall contains fibroblasts that are responsible for the synthesis of both type I and type III collagen. The deposition of LOX has been detected in an adult mouse aorta at the inner adventitial layer of the aortic wall. Moreover, further expression studies, a detailed immunohistochemical analysis, together with confocal and immunogold electron microscopy can determine if LOX crosslinks type I collagen, where LOXL appears in the developing aorta and whether either LOX or LOXL is responsible for crosslinking tropoelastin in the media.

For expression and co-localization studies, LOX and LOXL specific antibodies and antibodies specific to type I and III collagen are used to identify possible co-localization of these proteins. Furthermore, the *in situ* hybridization identifies the cells responsible for the synthesis of mRNAs encoding LOX and LOXL during the development of liver fibrosis and in the developing aorta. An elastin antibody is used to study colocalization of insoluble elastin with either LOX or LOXL.

Furthermore, the catalytic function of LOX and LOXL is analyzed using elastin as a substrate and peptides specific to type I and type III collagen. Differences in the catalytic activity of LOX have been shown when presented with different lysine-containing peptides (Kagan *et al.*, In: Molecular Biology and Pathology of Elastic Tissue, (Editors: Mecham and Roberts), Ciba Foundation Symposium Series, 1994). The potential substrate specificity for LOX and LOXL can be confirmed by demonstrating different Km values using lysine containing peptides from either type I or type III collagen, known to be involved in lysyl oxidase catalyzed cross-linking. In these catalytic assays, recombinant LOX and LOXL are used. Such recombinant LOX and LOXL are expressed from cDNA constructs that have been transfected into HeLa cells and immunopurified. These constructs can also have the FLAG epitope inserted at the C-terminus of both LOX and LOXL. Both the LOX and LOXL cDNAs encode the full length proteins. It has been shown that the catalytically active form of LOX is a 32 kD secreted protein produced by HeLa cells. LOXL, on the other hand, has been determined to be an extracellular matrix protein. Western blot analysis of tissue extracts of LOXL has revealed several proteins of different sizes, including a 33 kD polypeptide that the cDNA sequence can predict to be the secreted, active protein (Decitre *et al.* Lab. Invest. 78: 143-151, 1998). Therefore, the catalytically active of LOXL can be present in the media of transfected HeLa cell cultures.

Moreover, the activity of LOXL in the cell fractions, as well as in the media, are also measured. The presence of the FLAG label, the anti-FLAG antibody, together with LOXL specific antibody, can be employed to monitor LOXL processing and transport, and further determine the secreted active form of LOXL.

Full length LOX and LOXL expression constructs have been transfected into HeLa and ST6 fibroblasts, and the recombinant proteins can be detected using the anti-FLAG antibody. Furthermore, it has been established that the expressed LOX and LOXL are enzymatically active, confirming that the addition of the FLAG peptide at the C-

terminal end, closes to the catalytic site, does not interfere with the activity of LOX and LOXL.

Based on the differences in expression pattern, localization and on secondary structure predictions for the active sites, LOX and LOXL are believed to be extracellular matrix enzymes, that demonstrate different spatial and temporal expression patterns in cells and tissues with different extracellular substrate specificities. The studies on cell and tissue localization, co-localization with substrates and catalytic activity measurements on substrates, establish that LOX and LOXL have, indeed, different substrate specificity and preferred substrates.

It has also been determined that LOX and LOXL, rather than acting as separate amine oxidases, can act as synergistic isoforms of lysyl oxidase. The synergism of LOX and LOXL is determined using the recombinant LOX and LOXL expressed in HeLa cells together or in different ratios with different substrates. It has also been determined that during development, one of the LOX genes is expressed earlier, or is adapted to provide a rapid response to environmental changes, through different gene activation mechanisms, while the other LOX gene is expressed constitutively, to provide stable steady state levels for the corresponding mRNA, further providing insight into the developmentally determined differences in LOX and LOXL gene expression. It has also been identified that LOX and LOXL can also differ in other aspects such as the stability of the mRNAs encoding these proteins. As individually acting amine oxidases, or isoforms acting together, the results from the experments described here provide further insight into the mechanism of action of these two extracellular matrix lysyl oxidases in the assembly of fibrillar collagen and elastin.

EXAMPLE 6

LOXL2, LOXL3 and LOXL4 are membrane associated proteins

In initial experiments, it was shown that tissue expression of LOXL2 mRNA occurs in a wide range of tissues. As for the cell source, it shows trophoblasts that synthesize LOXL2 mRNA in the placenta.

Within tissues, LOXL2 protein appears around epithelial cells and endothelial cells in skin, in blood vessels, in hair follicles, in skeletal muscle and brain at sites where basement membrane is deposited. Furthermore, the immune-localization results show that LOXL2 can associate with the cell membrane. The detection of LOXL2 protein associatd with membrane may be the result of LOXL2 binding to a cell surface protein, receptor or structural protein(s) of the basement membrane.

Immunoprecipitation of the cell membrane fraction using LOXL2 antibodies is also performed and detects cell membrane associated LOXL2. Furthermore, in order to provide an *in vivo* correlation with results in intact tissue, the membrane associated localization of LOXL2 is also determined as a feature of cultured endothelial and/or epithelial cells by using either cultured endothelial or epithelial cells.

Based on the highly conserved domain structure, including the N-terminal SRCR domain, and the C-terminal catalytic and cytokine binding domains within LOXL3 and LOXL4 , it can be demonstrated that LOXL3 and LOXL4, like LOXL2, are membrane-associated proteins. Immune-localization of LOXL3 and LOXL4 proteins using specific antibodies in tissues, in fibroblasts and in endothelial cells can also be performed. Tissue distribution can be used to establish the presence of LOXL3 and LOXL4 relative to LOXL2 and the membrane associated localization of same based on domain homology.

LOXL3 and LOXL4 specific antibodies are designed against a region encoded by the human LOXL3 and LOXL4 cDNAs that have no homology to the other LOX-like proteins. Such antibodies are designed to domains of LOXL3 and LOXL4 that are highly

conserved between human and mouse, based on EST derived mouse LOXL3 and LOXL4 cDNA sequence analysis. Description of the LOXL3 and LOXL4 antibody generation is provided above.

A yeast two hybrid system is used to identify processing and activating agents, receptors, cell surface proteins, structural elements of the extracellular matrix and intracellular and intranuclear proteins that interact with either the cytokine binding C-terminal domain, the catalytic site and/or the N-terminal domain of LOX and LOXL, including the SRCR domains within LOXL2, LOXL3 and LOXL4.

Methods used for establishing that LOXL2, LOXL3 and LOXL4 are membrane associated proteins are described in detail below. Description of antibodies and immunohistology techniques are described above in EXAMPLE 5 section.

Membrane fraction immuno-precipitation: Normal HeLa cells or fibroblasts are unlabeled or radiolabeled with appropriate amino acids for 24-48 hours. Cells are then harvested and a membrane fraction is prepared as described (Wrenn *et al.* J. Biol. Chem. 238: 2280-2284, 1998). Immunoprecipitation is performed as described (Privitera *et al.* J. Biol. Chem. 273: 6319-6326, 1998). Briefly: 1 volume of membrane fraction is mixed in 2 volumes of IP buffer (0.3 ml NP-40, 0.3 g sodium deoxycholate, 20 mg sodium azide, 0.2 g bovine serum albumin, added to 100 ml of 10 mM tris-buffered saline [TBS] at pH 7.4), incubated for 1 hour at 4°C with 1/2 volume of normal rabbit serum and then, for 30 minutes, with two volumes of Protein A Sepharose beads (0.1 ml beads/ml buffer) to remove any unspecific immuno-complexes. The suspension is then centrifuged in a desktop microcentrifuge at 3000 rpm for 5 minutes and the supernatants used for immunoprecipitation. Aliquots of supernatants are incubated at 4°C with a polyclonal anti-LOX2, anti-LOXL3, or anti-LOXL4 antibody (2 pg/pl) for 2 hours. Two volumes of Protein A beads are subsequently added to the mixtures and incubated at 4°C for 1 hour with shaking. The tubes are then centrifuged for 10 minutes at 3000 rpm. The supernatant is removed and the pellet washed 3 times in IP buffer. The pellet is resuspended in an

appropriate volume of sample buffer, boiled for 5 minutes, resolved by 10% SDS-PAGE and stained with Silver Nitrate or Coommassie blue. Radiolabeled immunoprecipitated LOX2, LOXL3 or LOXL4 is detected by autoradiography.

Immunostaining of non-permeabilized cells: Cell cultures are washed in cold (4°C) PBS and then fixed in 0.5% paraformaldehyde (in PBS, pH adjusted to 7.4), washed in PBS (2 x 5 min) and blocked in 1% Bovine Serum Albumin (BSA in PBS)for 30 min. Antibody (anti-LOXL2, anti-LOXL3 or anti-LOXL4) is diluted 1:200 in PBS containing 0.1% BSA for 1 hour at room temperature. Cells are washed, 3 x 5 min in PBS with 0.1% BSA. A goat anti rabbit second antibody is added GAR-FITC diluted 1:100 in PBS containing 0.1% BSA for 1 hour in room temperature and washed, 2 x 5 min in PBS and mounted in Elvanol.

Immunostaining of cell ghosts: Cells in culture are washed in cold (4°C) PBS, 2x5 min and then incubated for 2 x 10 min in cold distilled water (this procedure will cause rupture of cell membranes). Cells are then fixed in 100% methanol and 3% paraformaldehyde, washed in PBS (2 x 5 min) and blocked in 1% Bovine Serum Albumin (BSA in PBS) for 30 min. First antibody is applied, (anti-LOXL2, LOXL3 or LOXL4) diluted 1:200 in PBS containing 0.1% BSA for 1 hour at room temperature. Cells are washed 3 x 5 min in PBS with 0.1% BSA, the second antibody is applied (Goat anti Rabbit) GAR-FITC diluted 1:100 in PBS containing 0.1% BSA for 1 hour at room temperature. Cells are washed 2 x 5 min in PBS and mounted in Elvanol.

EXAMPLE 7

Substrate activity and structure-function relationship of LOXL2, LOXL3 and LOXL4

In particular, this example describes an *in vitro* measurement of amine oxidase activity of immunopurified LOXL2 on synthetic peptide substrates. Such amine oxidase activity is shown to be inhibited by βAPN. Moreover, this example also describes the possibility that LOXL2 in cells that synthesize basement meembrane is functionally associated with basement membrane collagen type IV or other components of the

basement membrane, and further demonstrates that LOXL2 is structurally associated with the basement membrane and has a role in cell attachment.

A further aspect is that the N-terminal SPERACT/SRCR domains, the catalytic site and the overlapping cytokine binding site (that is actually a primitive fibronectin binding domain) within LOXL2, LOX3 and LOXL4 either directly (*e.g.*, through their amine oxidase activity, by crosslinking collagen type IV or other components of the extrcellular matrix or baement membrane) or indirectly (*e.g.*, through protein-protein interaction involving the cytokine binding site) fulfill significant functions in the assembly, structure or function of the basement membrane and/or the extrcellular matrix. Furthermore, one of these activities is the mechanistic basis for the reported role of LOXL2 in cell adhesion and the observed alterations in LOXL3 and LOXL4 expression observed in matastatic tumor cells.

The preliminary results provide evidence that LOXL2 is synthesized by cells that produce basement membrane and that LOXL2 appears in tissues co-localized with basement membrane. The overlapping catalytic and cytokine binding domains may have mutually exclusive functions. Thus, protein interactions at these sites can influence the conformation of LOXL2, LOXL3 and LOXL4 proteins and expose either the cytokine binding domain or the catalytric domain, and consequently play a direct role in cell adhesion or in substrate activation by LOXL2, LOXL3 and LOXL4.

In order to show that the catalytic domain of LOXL2, LOXL3 and LOXL4 is functional, the amine oxidase activity of these LOX-like proteins is measured on lysyl containing synthetic peptide substrate described by Kagan *et al.* (In: Molecular Biology and Pathology of Elastic Tissue, (Editors: Mecham and Roberts), Ciba Foundation Symposium Series, 1994). A full length LOXL2, or LOXL3 or LOXL4 cDNA is transfected in HeLa, CHO or embryonic kidney cells. The LOX-like proteins expressed in these cells are immuno-purified and isolated for use in activity assays. The detailed description of the activity assays is described in EXAMPLE 5.

It is also shown, by conformational analysis, that βAPN effectively inhibits the amine oxidase activity of these LOX-like proteins. These data provide further information on the conformation of the LOXL2, LOXL3 and LOXL4 catalytic sites.

Moreover, co-localizations suggest that the LOXL2 protein is the lysyl oxidase responsible for cross-linking collagen type IV.

Furthermore, the co-localization of the LOXL2 protein with the basement membrane components is detected using immunolabeling, confocal microscopy and immunogold labeling and electron microscopy on tissue sections. The data establish that LOXL2 directly binds to components of the basement membrane or to other extracellular proteins through the fibronectin type adhesive domain in LOXL2 that is known to fulfill a similar function in various adhesion proteins.

The experimental design to measure amine oxidase activity, the synthetic peptide substrates and the use of these peptide substrates in catalytic assays are described in EXAMPLES 5 and 6. Similarly, for immunopurification of LOXL2, isolation of the membrane bound LOXL2, immunostaining, immunogold labeling, confocal and electron microscopy are also described in EXAMPLE 5.

βAPN inhibition: The method used for determining the inhibition of amine oxidase activity of LOXL2 by LOX inhibitors, such as *βAPN*, phenylhydrazine, ethylendiamine, and diethyldithiocarbamate was described in detail by Tang *et al.*, (Connect. Tissue Res. 19: 93-103, 1989), and Kagan *et al.*, In: Molecular Biology and Pathology of Elastic Tissue, (Editors: Mecham and Roberts), Ciba Foundation Symposium Series, (1994). These assays are performed in triplicate as described in EXAMPLE 5.

LOXL2, LOXL3 and LOXL4 expression constructs: Generation of the LOXL2, LOXL3 and LOXL4 expression constructs have been described in EXAMPLE 5. The FLAG and GFP labels, expression vectors, transfections and cell expression constructs

have also been described earlier. LOXL2, LOXL3 and LOXL4 can be synthesized as a prepro-enzymes, similarly to LOX. Though the BMP1 processing site involving in the steps leading to LOX activation is present only within LOXL2, the other LOX-like proteins can be processed and activated by similar mechanisms. Indeed, Western blots of tissue and cell extracts using LOXL and LOXL2 antibodies detect several bands corresponding to these processed forms. LOXL2, LOXL3 or LOXL4 can be isolated using the 2M urea extraction method (Kagan *et al.* In: Molecular Biology and Pathology of Elastic Tissue, (Editors: Mecham and Roberts), Ciba Foundation Symposium Series, 1994) either from cell extracts, membrane fractions or cell culture media. Alternatively, a variety of detergents and previously described extraction procedures for membrane bound or membrane associated proteins can also be used for isolation of these proteins.

Immunohistochemistry: LOXL2, LOXL3 and LOXL4 specific antibodies and commercially available antibodies can be used to detect LOXL2, LOXL3 and LOXL4 and other laminin, collagen type IV and other extracellular matrix proteins, cell surface receptors, cellular and nuclear proteins that interact with LOX or the LOX-like amine oxidses. The detailed method for making LOXL2, LOXL3 and LOXL4 specific antibodies has been described in EXAMPLE 5.

Collagen type IV isolation: HT-1080 tumor cells can be used to generate labeled collagen type IV for the LOXL2 amine oxidase assays. These cells were successfully used earlier to describe the post-translational modifications in collagen type IV by lysyl hydroxylase. It has been reported that these cells secrete soluble collagen type IV into the medium. In contrast, Collagen type I and type III pro-collagens are not produced by these cells (Alitalo *et al.* Eur. J. Biochem. 109: 247-255, 1980). It has been determined interactions between the 7s domain of collagen type IV and LOXL2. Such interactions indicate that LOXL2 can stabilize the 7s domain of collagen type IV by lysine-derived cross-links (Kuhn *et al.*, FEBS Lett. 125: 123-128, 1981).

It has been established that free and/or membrane-associated LOXL2 has amine oxidase activity and that this activity is different from the catalytic activity of LOX due to structural differences between the two proteins. Furthermore, it has been established that in LOXL2, LOXL3 and LOXL4, the conformation of the cytokine binding domain can allow this domain to be involved in protein-protein interactions. These interactions can involve components of the basement membrane indicating the mechanistic basis for the reported LOXL2 function in cell adhesion or contribute to defining the specificity of substrate binding (Saito *et al.* J. Biol. Chem. 272: 8157-8160, 1997). Alternatively, the N-terminal SRCR domains play a role in protein interactions and consequently contribute to the cell adhesion function of the LOX-like proteins.

The catalytic domain in lysyl oxidases is embedded within a larger cytokine binding domain described by Kim *et al.*, (J. Biol. Chem. 270: 7176, 1995). The amino acids of the catalytic domain are strictly conserved in all LOX-like proteins, but there is divergence in the cytokine binding sites between LOX, LOXL, LOXL2, LOXL3 and LOXL4. While the catalytic site and its function, at least in LOX, is well characterized, these experiments provide insight into the role of the cytokine binding site of the LOX-like proteins.

The presence of the SRCR domains within LOXL2, LOXL3 and LOXL4 is a feature that distinguishes these LOX-like proteins from LOX and LOXL and establishes the novel function for these proteins. *In vivo*, LOXL2 has been detected to be associated with epidermal and endothelial cells. The membrane associated localization of LOXL2 has also been observed in cultured fibroblasts. The understanding of molecular and cellular processes associated with the membrane localization of LOXL2 has also been advanced.

Earlier studies have demonstrated that LOX has amine oxidase activity on a broad range of substrates including elastin, fibrillar collagens and lysine donors such as histone Hl. It has been established that crosslinking activity is a common feature of the conserved

catalytic domain of the LOX and LOX-like proteins, such as LOXL2, LOXL3 and LOXL4. Amine oxidase activity of LOXL2 is inhibited by βAPN, a well established specific inhibitor of LOX, has the same CH_3-C-(NH_2)-terminal group as the lysyl residue in some of the LOX substrates, but through its highly reactive nitril group, covalently reacts with neighboring residues irreversibly inhibiting LOX activity. Because of the small size of βAPN molecule, it can only occupy the catalytic site without interfering with other regions of the cytokine domain. Therefore, the efficiency of βAPN inhibition can provide additional information on the actual conformation of the active site in LOXL2 and indicate how this catalytic site is different from that of LOX. The absence or decrease of βAPN inhibition could be a strong indication of a variation in conformation within the active site of LOXL2 and consequently of different substrates or different functions for LOXL2.

It has been established that the catalytic and the cytokine binding-adhesive domains overlap and may function in a mutually exclusive manner. Once a substrate is bound to the active pocket, the larger adhesive domain is not accessible for interactions, or *vice versa*, interactions of the adhesive site with a structural protein can mask the catalytic site. The amine oxidase assays or the interacting protein co-localizations experiments can determine if LOXL2 is both active as a catalyst and as an adhesive protein. Furthermore, it can be determined whether membrane binding of LOXL2 is direct or indirect in its effect on either the catalytic or adhesive function. It can also be determined whether these functions are mutually exclusive and how these functions relate to the basement membrane and consequently, describe a mechanistic basis of the reported cell adhesion function of LOXL2.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SUMMARY OF SEQUENCES

Sequence ID NO:1 is the nucleic acid sequence of a cDNA encoding a human-derived LOX.

Sequence ID NO:2 is the deduced amino acid sequence of a cDNA encoding a human-derived LOX set forth in Sequence ID No. 1.

Sequence ID NO:3 is the nucleic acid sequence of a cDNA encoding a human-derived LOXL.

Sequence ID NO:4 is the deduced amino acid sequence of a cDNA encoding a human-derived LOXL set forth in Sequence ID No. 3.

Sequence ID NO:5 is the nucleic acid sequence of a cDNA encoding a human-derived LOXL2.

Sequence ID NO:6 is the deduced amino acid sequence of a cDNA encoding a human-derived LOXL2 set forth in Sequence ID No. 5.

Sequence ID NO:7 is the nucleic acid sequence of a cDNA encoding a human-derived LOXL3.

Sequence ID NO:8 is the deduced amino acid sequence of a cDNA encoding a human-derived LOXL3 set forth in Sequence ID No. 7.

Sequence ID NO:9 is the nucleic acid sequence of a cDNA encoding a human-derived LOXL4.

Sequence ID NO:10 is the deduced amino acid sequence of a cDNA encoding a human-derived LOXL4 set forth in Sequence ID No. 9.

Sequence ID NO:11 is the amino acid sequence of the copper binding domain of LOX or LOX-like polypeptide.

Sequence ID NO:12 is the amino acid sequence of the catalytic domain of LOX or LOX-like polypeptide.

Sequence ID NO:13 is the consensus sequence in the N-terminal modules of class I cytokine receptors.

Sequence ID NO:14 is the first 13 residues fit the Prosite pattern PS00241 of cytokine receptors.

Sequence ID NO:15 is the long extracellular SRCRconsensus sequence motif.

Sequence ID NO:16 is the short extracellular SRCR motif.

Sequence ID NO:17 is the amino acid sequence of the copper-binding domain of LOXL3 polypeptide.

Sequence ID NO:18 is the consensus sequence in the N-terminal telopeptides.

What is claimed is:

1. A substantially purified lysyl oxidase-like polypeptide encoded by a polynucleotide selected from the group consisting of:

(a) SEQ ID NO:7 or SEQ ID NO:7 wherein T can also be U, and nucleic acid sequences complementary thereto;

(b) fragments of (a) that are at least 15 bases in length and that will hybridize under stringent conditions to polynucleotide sequence of SEQ ID NO:7;

(c) SEQ ID NO:9 or SEQ ID NO:9 wherein T can also be U, and nucleic acid sequences complementary thereto; and

(d) fragments of (c) that are at least 15 bases in length and that will hybridize under stringent conditions to polynucleotide sequence of SEQ ID NO:9.

2. The polypeptide of claim 1 having an amino acid sequence substantially the same as set forth in SEQ ID NO:8 (LOXL3).

3. The polypeptide of claim 1 having an amino acid sequence of SEQ ID NO:8 (LOXL3).

4. The polypeptide of claim 1 having an amino acid sequence substantially the same as set forth in SEQ ID NO:10 (LOXL4).

5. The polypeptide of claim 1 having an amino acid sequence of SEQ ID NO:10 (LOXL4).

6. An isolated polynucleotide selected from the group consisting of:

(a) SEQ ID NO:7 or SEQ ID NO:7 wherein T can also be U, and nucleic acid sequences complementary thereto;

(b) fragments of (a) that are at least 15 bases in length and that will hybridize under stringent conditions to polynucleotide sequence of SEQ ID NO:7;

(c) SEQ ID NO:9 or SEQ ID NO:9 wherein T can also be U, and nucleic acid sequences complementary thereto; and

(d) fragments of (c) that are at least 15 bases in length and that will hybridize under stringent conditions to polynucleotide sequence of SEQ ID NO:9.

7. The isolated polynucleotide of claim 6 encoding a lysyl oxidase-like polypeptide having an amino acid sequence substantially the same as set forth in SEQ ID NO:8 or SEQ ID NO:10.

8. The isolated polynucleotide of claim 7, wherein said lysyl oxidase-like polypeptide comprises a copper binding domain with four Histidines (H) and a catalytic domain comprising Tyrosine (Y).

9. The isolated polynucleotide of claim 6 having nucleic acid sequence substantially the same as set forth in SEQ ID NO:7, or functional fragments thereof.

10. The isolated polynucleotide of claim 6, wherein said polynucleotide encodes a LOXL3 lysyl oxidase-like polypeptide having an amino acid sequence substantially the same as set forth in SEQ ID NO:8, or functional fragments thereof.

11. The isolated polynucleotide of claim 10, wherein said polynucleotide comprises a genomic polynucleotide comprising 14 exons and 13 introns, and maps to chromosome 2p13.3.

12. The isolated polynucleotide of claim 6 having nucleic acid sequence substantially the same as set forth in SEQ ID NO:9, or functional fragments thereof.

13. The isolated polynucleotide of claim 12, wherein said polynucleotide encodes a LOXL4 lysyl oxidase-like polypeptide having an amino acid sequence substantially the same as set forth in SEQ ID NO:10, or functional fragments thereof.

14. The isolated polynucleotide of claim 13, wherein said polynucleotide comprises a genomic polynucleotide comprising 13 exons and 12 introns, and maps to chromosome 10q24.

15. An expression vector containing a polynucleotide of claim 6.

16. A host cell containing a vector of claim 15.

17. A method for producing a lysyl oxidase-like polypeptide, said method comprising culturing a host cell a polynucleotide of claim 16 under conditions suitable for the expression of said polypeptide.

18. The method of claim 17 further comprising recovering said polypeptide from said host cell culture.

19. The method of claim 18, wherein said polypeptide is LOXL3 or LOXL4.

20. Antibodies which bind to the polypeptide of claim 1, or functional fragments thereof.

21. The antibodies of claim 20, wherein said antibodies are polyclonal or monoclonal antibodies.

22. A transgenic knockout mouse whose genome comprises a disruption of a lysyl oxidase or lysyl oxidase-like gene, wherein said disruption comprises insertion of a

transgene, and wherein said disruption results in exhibition of a disorder as compared to a wild-type mouse.

23. The transgenic knockout mouse of claim 22, wherein said disorder is a disorder of extracellular matrix materials, a cell migration disorder or a cell proliferative disorder.

24. The transgenic knockout mouse of claim 22, wherein said disorder is a skin disorder, a vascular system disorder, a skeletal development disorder, a neurological disorder, a hepatic system disorder, a copper-related disorder, a pulmonary system disorder, cancer, or a lathyrism disorder.

25. The transgenic knockout mouse of claim 22, wherein said mouse has homozygosity or heterozygosity of said disruption of an endogenous lysyl oxidase or lysyl oxidase-like gene.

26. A method for producing a transgenic knockout mouse exhibiting a lysyl oxidase or lysyl oxidase-like disorder, said method comprising:

(a) introducing a lysyl oxidase or lysyl oxidase-like transgene comprising a selectable marker sequence into a mouse embryonic stem cell;

(b) introducing said mouse embryonic stem cell into a mouse embryo;

(c) transplanting said embryo into a pseudopregnant mouse;

(d) allowing said embryo to develop to term; and

(e) identifying a transgenic mouse whose genome comprising a disruption of the endogenous lysyl oxidase or lysyl oxide-like gene, wherein said disruption resulting in an exhibition of lysyl oxidase or lysyl oxidase-like disorder as compared to a wild-type mouse.

27. The method of claim 26, wherein said transgenic mouse comprises homozygosity or heterozygosity of said disruption of an endogenous lysyl oxidase or lysyl oxidase-like gene.

28. A method for producing a transgenic mouse exhibiting a lysyl oxidase or lysyl oxidase-like disorder, said method comprising:

(a) transplanting a mouse embryo having mouse embryonic stem cells that contain a lysyl oxidase or lysyl oxidase-like transgene comprising a selectable marker sequence into a pseudopregnant mouse; and

(b) following development of said embryo to term, identifying a transgenic mouse whose genome comprising a disruption of an endogenous lysyl oxidse or lysyl oxidase-like gene, wherein said disruption resulting in said mouse exhibiting lysyl oxidase or lysyl oxidase-like disorder as compared to a wild type-mouse.

29. A method for identifying a compound that modulates lysyl oxidase or lysyl oxidase-like polypeptide activity, said method comprising:

(a) incubating said compound with a cell expressing a lysyl oxidase or lysyl oxidase-like polypeptide under conditions sufficient to permit said compound to interact with the cell;

(b) comparing a cellular response in said cell incubated with said compound with a cellular response of a cell not incubated with said compound, thereby identifying a compound that modulates lysyl oxidase or lysyl oxidase-like polypeptide activity.

30. The method of claim 29, wherein said cellular response is an amine oxidase activity.

31. The method of claim 29, wherein said lysyl oxidase or lysyl oxidase-like polypeptide activity is cross-linking extracellular matrix materials, modulating cell migration or modulating cell proliferation.

32. A method of treating a disorder associated with lysyl oxidase or lysyl oxidase-like polypeptide activity comprising administering to a subject in need thereof a therapeutically effective amount of a compound that modulates a lysyl oxidase or lysyl oxidase-like polypeptide activity.

33. The method of claim 32, wherein said disorder is a cell proliferative disorder, a cell migration disorder or a disorder related to extracellular matrix materials.

34. The method of claim 32, wherein said disorder is a skin disorder, a vascular system disorder, a skeletal development disorder, a neurological disorder, a hepatic system disorder, a copper-related disorder, a pulmonary system disorder, cancer, or a lathyrism disorder.

35. The method of claim 32, wherein said compound comprises an agonist or antagonist of lysyl oxidase or lysyl oxidase-like polypeptide activity.

36. A method of diagnosis of a subject having or at risk of having a cell proliferative disorder, a cell migration disorder or a disorder related to extracellular matrix materials, said method comprising detecting in the subject a level or activity of lysyl oxidase or lysyl oxidase-like polypeptide wherein a difference in the level or activity of lysyl oxidase or lysyl oxidase-like polypeptide in said subject from a level or activity of lysyl oxidase or lysyl oxidase-like polypeptide in a normal subject is indicative of a cell proliferative disorder, a cell migration disorder or a disorder related to extracellular matrix materials.

37. The method of claim 36, wherein said level or activity of a lysyl oxidase or lysyl oxidase-like polypeptide in said subject having or at risk of having a cell proliferative, a cell migration disorder or a disorder related to extracellular matrix materials disorder is

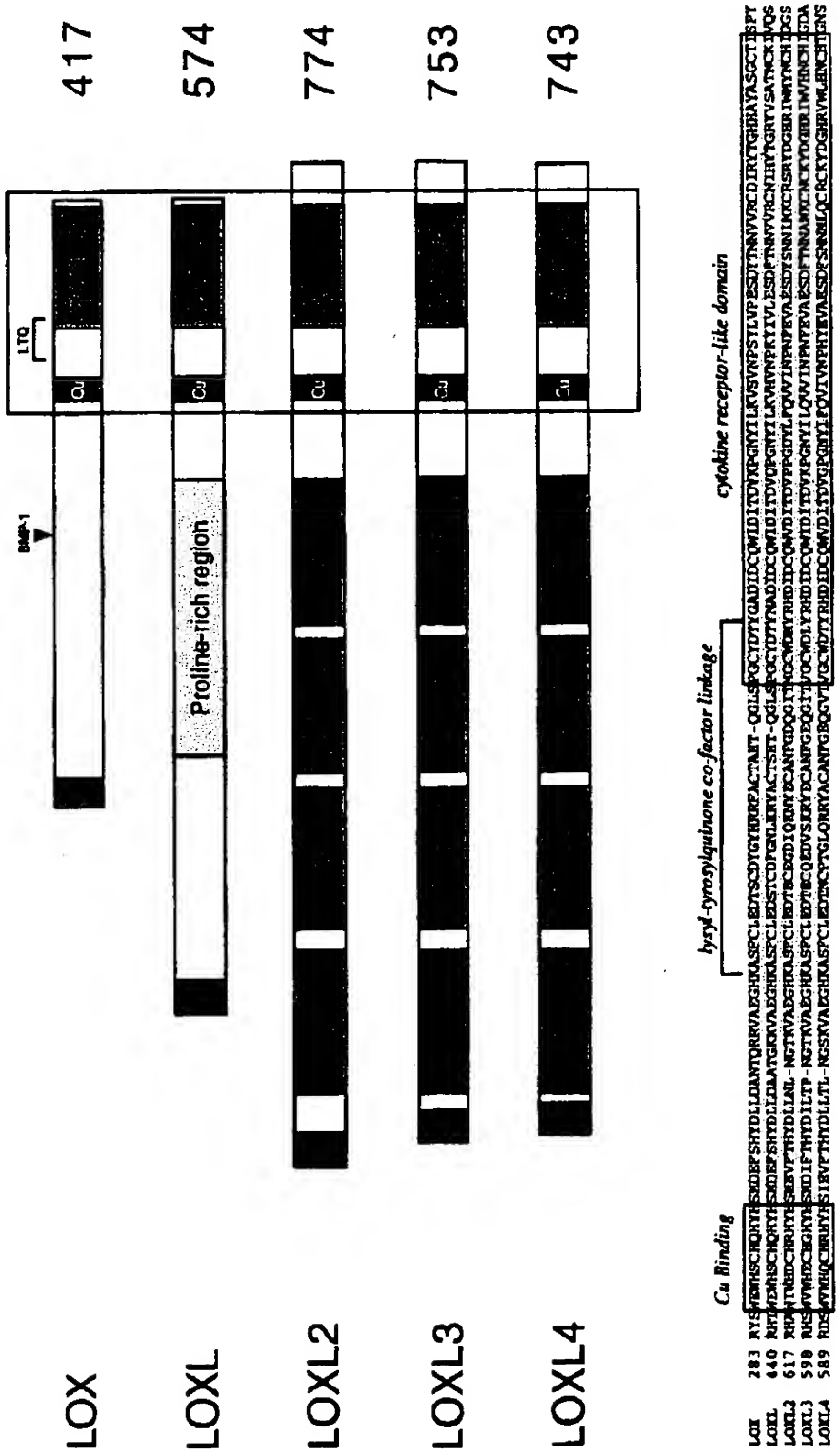
lower than said level or activity of lysyl oxidase or lysyl oxidase-like polypeptide in a normal subject.

38. The method of claim 36, wherein said level or activity of a lysyl oxidase or lysyl oxidase-like polypeptide in said subject having or at risk of having a cell proliferative, a cell migration disorder or a disorder related to extracellular matrix materials disorder is higher than said level or activity of lysyl oxidase or lysyl oxidase-like polypeptide in a normal subject.

39. A method of diagnosis of a subject having or at risk of having a skin disorder, a vascular system disorder, a skeletal development disorder, a neurological disorder, a hepatic system disorder, a copper-related disorder, a pulmonary system disorder, cancer, or a lathyrism disorder comprising detecting in the subject a level or activity of a lysyl oxidase or lysyl oxidase-like polypeptide, wherein a difference in the level or activity of lysyl oxidase or lysyl oxidase-like polypeptide in said subject from a level or activity of lysyl oxidase or lysyl oxidase-like polypeptide in a normal subject is indicative of a skin disorder, a vascular system disorder, a skeletal development disorder, a neurological disorder, a hepatic system disorder, a copper-related disorder, a pulmonary system disorder, cancer, or a lathyrism disorder.

40. The method of claim 39, wherein said level or activity of lysyl oxidase or lysyl oxidase-like polypeptide in said subject having or at risk of having a cell proliferative, a cell migration disorder or a disorder related to extracellular matrix materials disorder is lower than the level or activity of lysyl oxidase or lysyl oxidase-like polypeptide in a normal subject.

41. The method of claim 39, wherein said level or activity of lysyl oxidase or lysyl oxidase-like polypeptide in said subject having or at risk of having a cell proliferative, a cell migration disorder or a disorder related to extracellular matrix materials disorder is higher than the level or activity of lysyl oxidase or lysyl oxidase-like polypeptide in a normal subject.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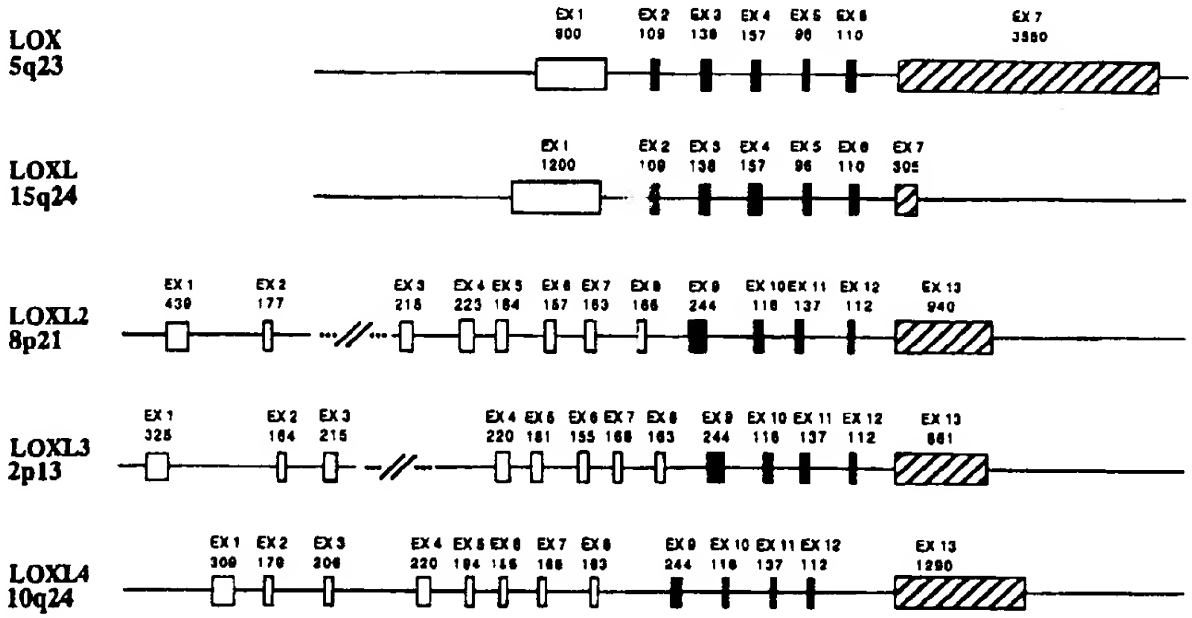
LOXL
LOXL2
LOXL3
LOXL4
417
574
774
753
743
LTQ
Cu
BMP-1
Proline-rich region
lysyl-tyrosylquinone co-factor linkage
cytokine receptor-like domain
Cu Binding

Figure 1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EX 3 138
EX 4 157
EX 5 96
EX 6 110
EX 7 3580
LOXL
15q24
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EX 2 109
EX 3 138
EX 4 157
EX 5 96
EX 6 110
EX 7 305
LOXL2
8p21
EX 1 439
EX 2 177
EX 3 218
EX 4 223
EX 5 184
EX 6 157
EX 7 163
EX 8 166
EX 9 244
EX 10 116
EX 11 137
EX 12 112
EX 13 940
LOXL3
2p13
EX 1 325
EX 2 164
EX 3 215
EX 4 220
EX 5 181
EX 6 155
EX 7 168
EX 8 163
EX 9 244
EX 10 116
EX 11 137
EX 12 112
EX 13 661
LOXL4
10q24
EX 1 309
EX 2 179
EX 3 206
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EX 12 112
EX 13 1290

FIGURE 2

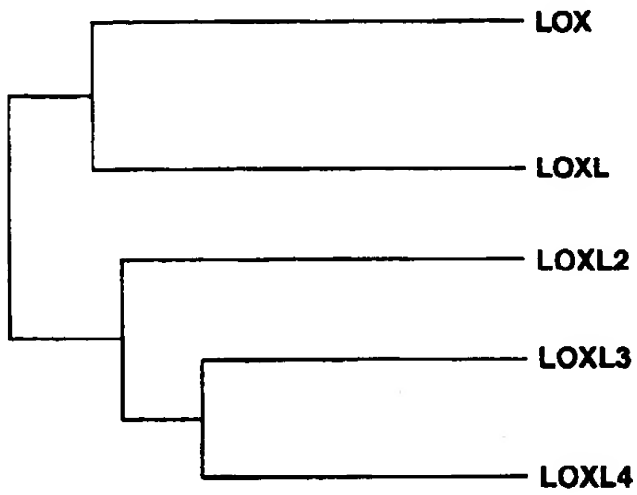


Figure 3

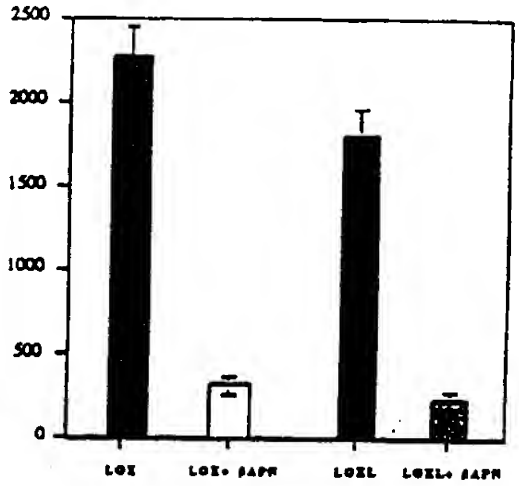
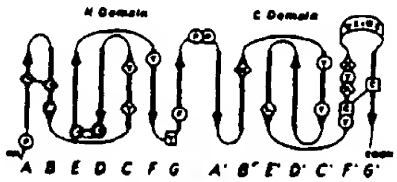
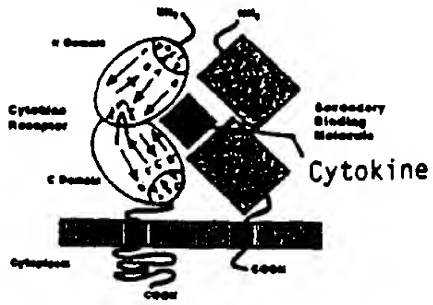


FIGURE 4

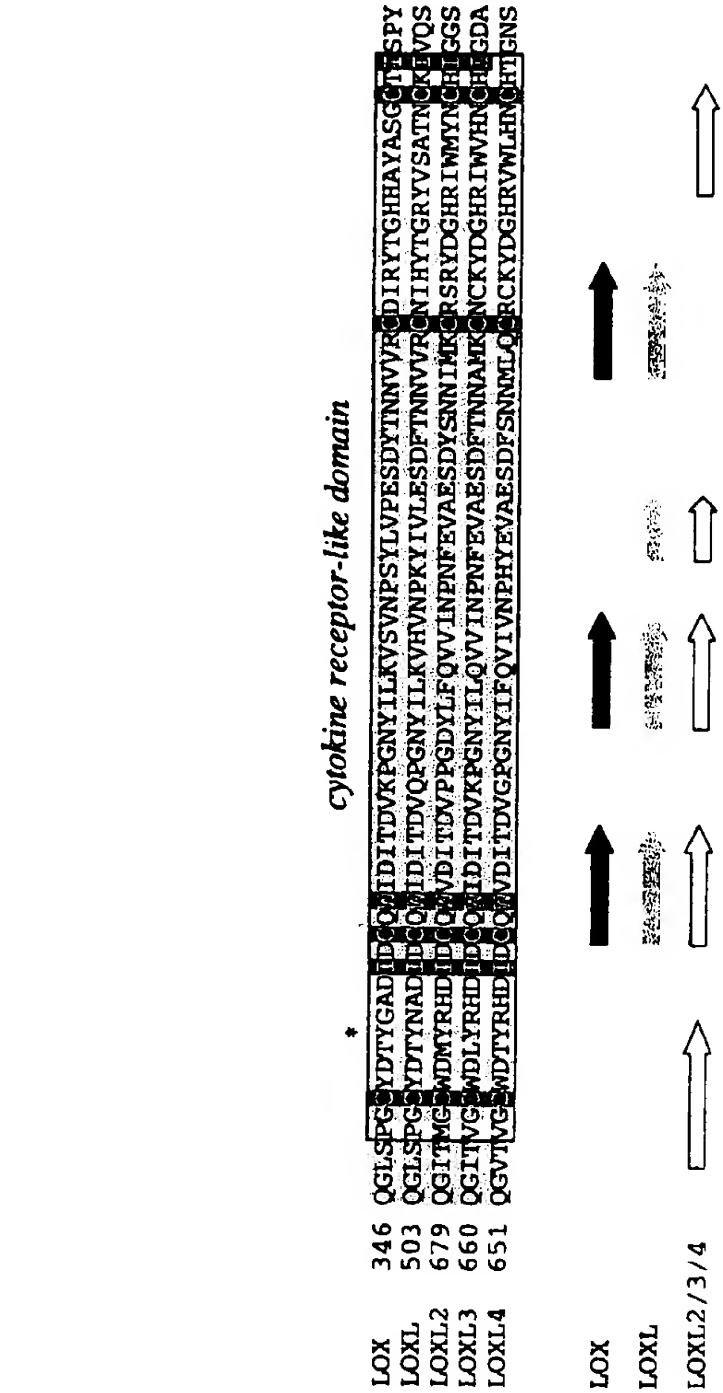


5A



5B

FIGURE 5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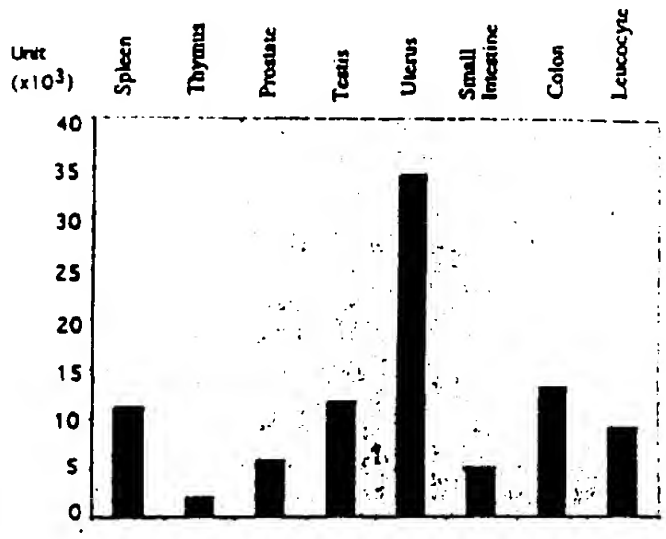
LOXL 503
LOXL2 679
LOXL3 660
LOXL4 651
LOX
LOXL
LOXL2/3/4

Figure 6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(x10³)
Spleen
Thymus
Prostate
Testis
Uterus
Small Intestine
Colon
Leucocyte
40
35
30
25
20
15
10
5
0

FIGURE 7

SEQUENCE LISTING

SEQ ID NO:1 LOX cDNA sequence

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SEQ ID NO:2 LOX amino acid sequence

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SEQ ID NO: 3LOXL cDNA sequence

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SEQ ID NO: 4LOXL amino acid sequence

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SEQ ID NO:5 LOXL2 cDNA sequence (human)

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SEQ ID NO:6 LOXL2 amino acid sequence (human)

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SEQ ID NO:7 LOXL3 cDNA sequence (human)

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SEQ ID NO:8 LOXL3 amino acid sequence (human)

MRPVSVWQWS PWGLLLCLLC SSCLGSPSPS TGPEKKAGSQ GLRFRLAGFP RKPYEGRVEI
QRAGEWGTIC DDDFTLQAAH ILCRELGFTE ATGWTHSAKY GPGTGRIWLD NLSCSGTEQS
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DAGVRCNLPY TGAETRIRLS GGRSQHEGRV EVQIGGPGPL RWGLICGDDW GTLEAMVACR
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DTECQEDVSK RYECANFGEQ GITVGCWDLY RHDIDCQWID ITDVKPGNYI LQVVINPNFE
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SEQ ID NO:9 LOXL4 cDNA Sequence (Human)

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SEQ ID NO:10 LOXL4 amino acid sequence (Human) (Length: 162 aa)

MAWSPPATLFLFLLLLGQPPPSRPQSLGTTKLRLVGPESKPEEGRLEVLHQGQWGTVCDDNFA
IQEATVACRQLGFEAALTWAHSAKYGQGEGPIWLDNVRCVGTESSLDQCGSNGWGVSDCSH
SEDVGVICHPRRHRGYLSETVSNALGPQGRRLEEVRLKPILASAKQHSPVTEGAVEVKYEGHW
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QVQVAPARGKLRPACPGGMHAVVSCVAGPHFRPPKTKPQRKGSWAEEPRVRLRSGAQVGEG
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QCQRHGPVHCSHGGGRFLAGVSCMDSAPDLVMNAQLVQETAYLEDRPLSQLYCAHEENCLS
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LNGSKVAEGHKASFCLEDTNCPTGLQRRYACANFGEQGVTVGCWDTYRHDIDCQWVDITDV
GPGNYIFQVIVNPHYEVAESDFSNNMLQCRCKYDGHRVWLHNCHTGNSYPANAELSLEQEQR
LRNNLI

SEQ ID NO. 11 Amino acid sequence of the copper-binding domain of LOX or LOX-like polypeptide

WEWHSCHQHYHSM

Sequence ID NO. 12 Amino acid sequence of the catalytic domain of LOX or LOX-like polypeptide

DIDCQWWIDITDVXPGNY

Sequence ID NO. 13 Consensus sequence in the N-terminal modules of class I cytokine receptors

C-X_9-C-X-W-X_{26-32}-C X_{10-13}-C

Sequence ID NO. 14 First 13 residues fit the Prosite pattern PS00241 of cytokine receptors

C-[LVDYR]-X(7,8)-[STIVDN]-C-X-W

Sequence ID NO. 15 Long extracellular SPERACT-receptor consensus sequence motif

GAY IGE GRV EVL KNG EWG TVC DDK WDL VSA SVV GRE LG

Sequence ID NO. 16Short extracellular SPERACT-receptor motif

CSHSQDAGVRC

Sequence ID NO. 17 Amino acid sequence of the copper-binding domain of LOXL3 polypeptide.

WVWHECHGHYHSM

Sequence ID NO. 18 Consensus sequence in the N-terminal telopeptides

X-Asp-J-Lys-Z